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(54) Title: <b>INDUCIBLE HERBICIDE RESISTANCE</b>			
(57) Abstract			
<p>The invention relates to DNA constructs which are capable of conferring on a plant inducible resistance to a herbicide. The inducible effect may be achieved by using a gene switch such as the <i>alcA/alcR</i> switch derived from <i>A. nidulans</i>. The invention relates in particular to inducible resistance to the herbicide N-phosphonomethyl glycine (glyphosate) and its salts.</p>			

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## INDUCIBLE HERBICIDE RESISTANCE

The present invention relates to DNA constructs and plants incorporating them. In particular it relates to promoter sequences for the expression of genes which confer herbicide resistance on plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from *Streptomyces hydroscopicus*. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from *Arabidopsis*, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter.

We propose a system where genes conferring herbicide tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide resistance genes. This may be a particular problem as early stages of growth where high levels of transgene product may directly interfere with normal development. Alternatively high levels of expression of herbicide resistance genes may cause a metabolic drain for plant resources.
2. The expression of herbicide resistance genes in an inducible manner allows the herbicide in question to be used to control volunteers if the activating chemical is omitted during treatment.
3. The use of an inducible promoter to drive herbicide resistance genes will reduce the risk of resistant weed species becoming a major problem. If resistance genes were passed onto weed species from related crops, control could still be achieved with the herbicide in the absence of inducing chemical. This would particularly be relevant if the tolerance gene confirmed resistance to a total vegetative control herbicide which would be used (with no inducing chemical) prior to sowing the crop and potentially after the crop has been harvested. For example, it can be envisaged that herbicide

resistance in cereals, such as wheat, might outcross into the weed wild oats or that herbicide resistance in oil seed rape or canola could be transferred to wild brassicas thus conferring herbicide resistance to these already troublesome weeds. A further example is that the inducible expression of herbicide resistance in sugar beet will 5 reduce the risk of wild sugar beet becoming a problem.

Several gene regulation systems (gene switches) are known and may be used for conferring inducible herbicide resistance on plants. Many such gene switches are described in the review by Gatz (Current Opinion in Biotechnology (1996) 7, 168-172) and include systems such as the tetracycline repressor gene switch, the Lac repressor system, copper 10 inducible systems such as that based on ACE 1, salicylic acid inducible promoters including the PR-1a system and systems based on steroid hormones such as the glucocorticoid, progesterone and oestrogen receptor systems. Modifications of the glucocorticoid receptor systems which include the GAL 4 binding domain from yeast and the VP16 activator are described by Aoyama *et al*, *The Plant Cell*, (1995) 7, 1773-1785 and it is envisaged that 15 similar systems may be based on, for example insect steroid hormones rather than on mammalian steroid hormones. Indeed, a system based on the ecdysone receptor of *Heliothis virescens* has recently been described. Benzene sulphonamide gene switching systems are also known (Hershey *et al*, *Plant Mol. Biol.*, 17, 679-690 (1991) as are systems based on the alcR protein from *Aspergillus nidulans* and glutathione S-transferase promoters.

20 Several genes which confer herbicide resistance are also known, for example, one herbicide to which resistance genes have been described and which is extremely widely used is N-phosphonomethyl-glycine (glyphosate) and its agriculturally acceptable salts including the isopropylamine, trimethylsulphonium, sodium, potassium and ammonium salts.

25 In a first aspect of the present invention there is provided a chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.

Any herbicide resistance gene may be used but genes which confer resistance to N-phosphonomethyl-glycine or salts or derivatives thereof are especially preferred.

30 Several inducible promoters may be used to confer the inducible resistance and these include any of those listed above.

However, a particularly useful gene switch for use in this area is based on the *alcR* regulatory protein from *Aspergillus nidulans* which activates genes expression from the *alcA* promoter in the presence of certain alcohols and ketones. This system is described in our International Patent Publication No. WO93/21334 which is incorporated herein by reference.

5        The *alcA/alcR* gene activation system from the fungus *Aspergillus nidulans* is also well characterised. The ethanol utilisation pathway in *A. nidulans* is responsible for the degradation of alcohols and aldehydes. Three genes have been shown to be involved in the ethanol utilisation pathway. Genes *alcA* and *alcR* have been shown to lie close together on linkage group VII and *aldA* maps to linkage group VIII (Pateman JH *et al*, 1984, Proc. Soc. 10 Lond, B217:243-264; Sealy-Lewis HM and Lockington RA, 1984, Curr. Genet, 8:253-259). Gene *alcA* encodes ADHI in *A. nidulans* and *aldA* encodes AldDH, the second enzyme responsible for ethanol utilisation. The expression of both *alcA* and *aldA* are induced by ethanol and a number of other inducers (Creaser EH *et al*, 1984, Biochemical J, 255:449-454) via the transcription activator *alcR*. The *alcR* gene and a co-inducer are responsible for the 15 expression of *alcA* and *aldA* since a number of mutations and deletions in *alcR* result in the pleiotropic loss of ADHI and aldDH (Felenbok B *et al*, 1988, Gene, 73:385-396; Pateman *et al*, 1984; Sealy-Lewis & Lockington, 1984). The ALCR protein activates expression from *alcA* by binding to three specific sites in the *alcA* promoter (Kulmberg P *et al*, 1992, J. Biol. Chem, 267:21146-21153).

20        The *alcR* gene was cloned (Lockington RA *et al*, 1985, Gene, 33:137-149) and sequenced (Felenbok *et al*, 1988). The expression of the *alcR* gene is inducible, autoregulated and subject to glucose repression mediated by the CREA repressor (Bailey C and Arst HN, 1975, Eur. J. Biochem, 51:573-577; Lockington RA *et al*, 1987, Mol. Microbiology, 1:275-281; Dowzer CEA and Kelly JM, 1989, Curr. Genet, 15:457-459; Dowzer CEA and Kelly 25 JM, 1991, Mol. Cell. Biol, 11:5701-5709). The ALCR regulatory protein contains 6 cysteines near its N terminus co-ordinated in a zinc binuclear cluster (Kulmberg P *et al*, 1991, FEBS Letts, 280:11-16). This cluster is related to highly conserved DNA binding domains found in transcription factors of other ascomycetes. Transcription factors GAL4 and LAC9 have been shown to have binuclear complexes which have a cloverleaf type structure 30 containing two Zn(II) atoms (Pan T and Coleman JE, 1990, Biochemistry, 29:3023-3029; Halvorsen YDC *et al*, 1990, J. Biol. Chem, 265:13283-13289). The structure of ALCR is

similar to this type except for the presence of an asymmetrical loop of 16 residues between Cys-3 and Cys-4. ALCR positively activates expression of itself by binding to two specific sites in its promoter region (Kulmberg P *et al*, 1992, *Molec. Cell. Biol.*, 12:1932-1939).

5 The regulation of the three genes, *alcR*, *alcA* and *aldA*, involved in the ethanol utilisation pathway is at the level of transcription (Lockington *et al*, 1987; Gwynne D *et al*, 1987, *Gene*, 51:205-216; Pickett *et al*, 1987, *Gene*, 51:217-226).

There are two other alcohol dehydrogenases present in *A. nidulans*. ADHII is present in mycelia grown in non-induced media and is repressible by the presence of ethanol. ADHII is encoded by *alcB* and is also under the control of *alcR* (Sealy-Lewis & Lockington, 1984).  
10 A third alcohol dehydrogenase has also been cloned by complementation with a *adh-* strain of *S. cerevisiae*. This gene *alcC*, maps to linkage group VII but is unlinked to *alcA* and *alcR*. The gene, *alcC*, encodes ADHIII and utilises ethanol extremely weakly (McKnight GL *et al*, 1985, *EMBO J.*, 4:2094-2099). ADHIII has been shown to be involved in the survival of *A. nidulans* during periods of anaerobic stress. The expression of *alcC* is not repressed by the  
15 presence of glucose, suggesting that it may not be under the control of *alcR* (Roland LJ and Stromer JN, 1986, *Mol. Cell. Biol.*, 6:3368-3372).

In summary, *A. nidulans* expresses the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene *alcA* only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the *alcR* gene and 20 constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the *alcA* gene. The regulator protein also stimulates expression of itself in the presence of inducer. This means that high levels of the ADH1 enzyme are produced under inducing conditions (i.e. when alcohol or ketone are present). Conversely, the *alcA* gene and its product, ADH1, are not expressed in the absence of inducer.  
25 Expression of *alcA* and production of the enzyme is also repressed in the presence of glucose.

Thus the *alcA* gene promoter is an inducible promoter, activated by the *alcR* regulator protein in the presence of inducer (i.e. by the protein/alcohol or protein/ketone combination). The *alcR* and *alcA* genes (including the respective promoters) have been cloned and sequenced (Lockington RA *et al*, 1985, *Gene*, 33:137-149; Felenbok B *et al*, 1988, *Gene*, 30 73:385-396; Gwynne *et al*, 1987, *Gene*, 51:205-216).

Alcohol dehydrogenase (*adh*) genes have been investigated in certain plant species. In maize and other cereals they are switched on by anaerobic conditions. The promoter region of *adh* genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the *alcR* regulator protein has been found in any plant. Hence the *alcR/alcA* type of gene regulator system is not known in plants.

Constitutive expression of *alcR* in plant cells does not result in the activation of endogenous *adh* activity.

According to a second aspect of the invention, there is provided a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcR* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene.

The inducible promoter is preferably derived from the *alcA* gene promoter but may, alternatively be derived from *alcR*, *aldA* or other *alcR*-induced genes.

We have found that the *alcA/alcR* switch is particularly suited to drive herbicide tolerance genes for at least the following reasons.

1. The *alcA/alcR* switch has been developed to drive high levels of gene expression. In addition, the regulatory protein *alcR* is preferably driven from a strong constitutive promoter such as polyubiquitin. High levels of induced transgene expression, comparable to that from a strong constitutive promoter, such as 35 CaMV, can be achieved.
2. If a gene switch is to be used in a situation where the activating chemical is applied simultaneously with the herbicide, a rapid elevation in the levels of herbicide resistance gene is required. Figure 1 reveals a time course of marker gene expression (CAT) following application of inducing chemical. This study shows a rapid increase (2 hours) of CAT expression following foliar application of inducing chemical. The immediate early kinetics of induction are brought about by expressing the regulatory protein in constitutive manner, therefore no time lag is encountered while synthesis of transcription factors takes place. In addition we have chosen a simple two component system which does not rely on a complex signal transduction system.

3. We have tested the specificity of *alcA/alcR* system with a range of solvents used in agronomic practice. A hydroponic seedling system revealed that ethanol, butan-2-ol and cyclohexanone all gave high levels of induced reporter gene expression (Figure 2). In contrast when the alcohols and ketones listed in Table 1 in which are used in

5 agronomic practice were applied as a foliar spray only ethanol gave high levels of induced reporter gene activity (Figure 3).

Table 1

1.	Isobutyl methyl ketone	13.	acetyl acetone
2.	Fenchone	14.	JF5969 (cyclohexanone)
3.	2-heptanone	15.	N-methyl pyrrolidone
4.	Di-isobutyl ketone	16.	polyethylene glycol
5.	5-methyl-2-hexanone	17.	propylene glycol
6.	5-methylpentan-2,4-diol	18.	acetophenone
7.	ethyl methyl ketone	19.	JF4400 (methylcyclohexanone)
8.	2-pentanone	20.	propan-2-ol
9.	glycerol	21.	butan-2-ol
10.	$\gamma$ -butyrolactone	22.	acetone
11.	diacetone alcohol	23.	ethanol
12.	tetrahydrofurfuryl alcohol	24.	dH <sub>2</sub> O

10 This is of significance since illegitimate induction of transgenes will not be encountered by chance exposure to formulation solvents. Ethanol is not a common component of agrochemical formulations and therefore with appropriate spray management can be considered as a specific inducer of the *alc A / R* gene switch in a field situation.

15 4. A range of biotic and abiotic stresses for example pathogen infection, heat, cold, drought, wounding, flooding have all failed to induce the *alcA / alcR* switch. In addition a range of non-solvent chemical treatments for example salicylic acid, ethylene, abscisic acid, auxin, gibberellic acid, various agrochemicals, all failed to induce the *alcA / alcR* system.

The first promoter may be constitutive or tissue-specific, developmentally-programmed or even inducible. The regulator sequence, the *alcR* gene, is obtainable from *Aspergillus nidulans*, and encodes the *alcR* regulator protein.

5 The inducible promoter is preferably the *alcA* gene promoter obtainable from *Aspergillus nidulans* or a "chimeric" promoter derived from the regulatory sequences of the *alcA* promoter and the core promoter region from a gene promoter which operates in plant cells (including any plant gene promoter). The *alcA* promoter or a related "chimeric" promoter is activated by the *alcR* regulator protein when an alcohol or ketone inducer is applied.

10 The inducible promoter may also be derived from the *aldA* gene promoter, the *alcB* gene promoter or the *alcC* gene promoter obtainable from *Aspergillus nidulans*.

15 The inducer may be any effective chemical (such as an alcohol or ketone). Suitable chemicals for use with an *alcA/alcR*-derived cassette include those listed by Creaser *et al* (1984, Biochem J, 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, acetone, butan-2-ol, 3-oxobutyric acid, propan-2-ol, ethanol.

The gene expression cassette is responsive to an applied exogenous chemical inducer enabling external activation of expression of the target gene regulated by the cassette. The expression cassette is highly regulated and suitable for general use in plants.

20 The two parts of the expression cassette may be on the same construct or on separate constructs. The first part comprises the regulator cDNA or gene sequence subcloned into an expression vector with a plant-operative promoter driving its expression. The second part comprises at least part of an inducible promoter which controls expression of a downstream target gene. In the presence of a suitable inducer, the regulator protein produced by the first part of the cassette will activate the expression of the target gene by stimulating the inducible promoter in the second part of the cassette.

25 In practice the construct or constructs comprising the expression cassette of the invention will be inserted into a plant by transformation. Expression of target genes in the construct, being under control of the chemically switchable promoter of the invention, may then be activated by the application of a chemical inducer to the plant.

30 Any transformation method suitable for the target plant or plant cells may be employed, including infection by *Agrobacterium tumefaciens* containing recombinant Ti

plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained

5 in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

10 The invention further provides a plant cell containing a gene expression cassette according to the invention. The gene expression cassette may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

15 The invention further provides a method for controlling plant gene expression comprising transforming a plant cell with a chemically-inducible plant gene expression cassette which has a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcA* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the

20 inducer causes expression of the target gene.

25 This strategy of inducible expression of herbicide resistance can be achieved with a pre-spray of chemical activator or in the case of slow acting herbicides, for example N-phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be added as a tank mix simultaneously with the herbicide.

This strategy can be adopted for any resistance conferring gene/corresponding herbicide combination. For example, the *alcA/alcR* gene switch can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent Publication No WO90/08826), which confers resistance to chloroacetanilide herbicides such as acetochlor, metolachlor and alachlor.
- 30 2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide commonly known as glufosinate.

3. Acetolactate synthase gene mutants from maize (see our International Patent Publication No WO90/14000) and other genes, which confer resistance to sulphonyl urea and imadazolones.
4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377 in the name of Monsanto Company); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201 in the name of Monsanto Company); and genes which are involved in the expression of CPLyase, Various further preferred features and embodiments of the present invention will now be described in the non-limiting examples set out below and with reference to the drawings in which:

15 Figure 1 illustrates the time course of marker gene expression (CAT) following application of inducing chemical;

Figure 2 illustrates the levels of induced reporter gene expression on root drenching with a range of solvents;

Figure 3 illustrates the levels of induced reporter gene activity when the chemicals listed in Table 1 were applied as a foliar spray;

20 Figure 4 illustrates the production of the 35S regulator construct by ligation of *alcR* cDNA into pJRI.

Figure 5 illustrates the production of the reporter construct;

Figure 6 is a summary of the cassettes and specific plant transformation constructs;

25 Figure 7 illustrates the chloroplast transit sequence 1 from *Arabidopsis* RUBISCO (CPT 1);

Figure 8 shows the sequence of plasmid pMJB1;

Figure 9 is a map of plasmid pJRIi;

Figure 10 illustrates the chloroplast transit sequence CTP2 from EPSPS class I gene from *Petunia hybrida*;

30 Figure 11 is a map of plasmid pUB-1;

Figure 12 is a map of plasmid pMF6;

Figure 13 is a map of plasmid pIE109 in which the numbers are in base pairs (not to scale) and the following abbreviations are used:

ADH <sub>i</sub>	Alcohol dehydrogenase from maize;
5 PAT	Phosphinothricin acetyl transferase (Basta resistance gene);
AMP	Ampicillin resistance gene;
CaMV 35S	Cauliflower mosaic virus 35S promoter;
nos Poly A	Nopaline synthase poly A region;
ori	ColE1 origin of replication from pUC

10 Figure 14 is a map of plasmid pMV1 in which the numbers are in base pairs (not drawn to scale) and the abbreviations are as for Figure 13 with the following additional abbreviations:

UBQ <sub>p</sub>	Maize ubiquitin promoter;
UBQ <sub>i</sub>	Maize ubiquitin intron;
15 nos	Nopaline synthase 3' terminator;
CZP1 GOX	Chloroplast transit peptide - glyphosate oxidase sequence;
CZP2 GPSPS	Chloroplast transit peptide - EPSP synthetase sequence;

Figure 15 shows the preparation of plasmid pUC4 by ligation of pAr3 and pBSSK;

Figure 16 is a map of plasmid pMV2 in which the numbers are in base pairs (not

20 drawn to scale) and the abbreviations are as for Figure 14 with the following additional abbreviations:

AlcA	<i>Aspergillus nidulans alcA</i> promoter;
AlcR	<i>Aspergillus nidulans alcR</i> promoter;

Figure 17 is a map of plasmid pDV1-pUC;

25 Figure 18 is a map of plasmid pDV2-pUC;

Figure 19 is a map of plasmid pDV3-Bin;

Figure 20 is a map of plasmid pDV4-Bin; and

Figure 21 is a western Blot showing the expression of EPSPS and GOX in transformants.

## EXAMPLES

We have chosen to exemplify the *alcA/alcR* gene switch with genes conferring resistance to glyphosate. The switch will be used to drive inducible expression of glyphosate oxidase (GOX) in plants. Switchable GOX has been expressed alone or in conjunction with 5 constitutive expression of 5-enol-pyruvylshikimate 3-phosphate (EPSPS) CP4. Constructs have been optimised for expression in monocotyledonous and dicotyledonous crop species.

### EXAMPLE 1

#### **Production Of The *alcR* Regulator Construct.**

The *alcR* genomic DNA sequence has been published, enabling isolation of a sample of *alcR* cDNA.

The *alcR* cDNA was cloned into the expression vectors pJR1(pUC). pJR1 contains the Cauliflower Mosaic Virus 35S promoter. This promoter is a constitutive plant promoter and will continually express the regulator protein. The *nos* polyadenylation signal is in the expression vector.

Figure 4 illustrates the production of the 35S regulator construct by ligation of *alcR* cDNA into pJR1. Partial restriction of the *alcR* cDNA clone with *Bam*HI was followed by electrophoresis in an agarose gel and the excision and purification of a 2.6 Kb fragment. The fragment was then ligated into the pJR1 vector which had been restricted with *Bam*HI and phosphatased to prevent recircularisation. The *alcR* gene was thus placed under control of the CaMV 35S promoter and the *nos* 3' polyadenylation signal in this "35S-*alcR*" construct.

### EXAMPLE 2

#### **Production Of The *alcA*-CAT Reporter Construct Containing The Chimeric Promoter.**

The plasmid pCaMVCN contains the bacterial chloramphenicol transferase (CAT) reporter gene between the 35S promoter and the *nos* transcription terminator (the "35S-CAT" construct).

The *alcA* promoter was subcloned into the vector pCaMVCN to produce an "alcA-CAT" construct. Fusion of part of the *alcA* promoter and part of the 35S promoter created a chimeric promoter which allows expression of genes under its control.

Figure 5 illustrates the production of the reporter construct. The *alcA* promoter and the 35S promoter have identical TATA boxes which were used to link the two promoters together using a recombinant PCR technique: a 246 bp region from the *alcA* promoter and

the 5' end of the CAT gene from pCaMVCN (containing part of the -70 core region of the 35S promoter) were separately amplified and then spliced together using PCR. The recombinant fragment was then restriction digested with *Bam*HI and *Hind*III. The pCaMVCN vector was partially digested with *Bam*HI and *Hind*III, then electrophoresed so 5 that the correct fragment could be isolated and ligated to the recombinant fragment.

The ligation mixtures were transformed into *E. coli* and plated onto rich agar media. Plasmid DNA was isolated by miniprep from the resultant colonies and recombinant clones were recovered by size electrophoresis and restriction mapping. The ligation junctions were sequenced to check that the correct recombinants had been recovered.

10 **EXAMPLE 3**

**Glyphosate Resistance Constructs**

A summary of the cassettes and specific plant transformation constructs is shown in Figure 6.

**Dicot Vector 1**

15 Vector 1 is a constitutive control plasmid containing the glyphosate oxidase gene (GOX) fused to the chloroplast transit sequence 1 from *Arabidopsis* RUBISCO (CPT 1) (Figure 7) driven by the enhanced 35S CaMV promoter (ES) and the TMV omega translational enhancer sequence (TMV). Vector 1 utilizes the nopaline synthase terminator (nos). The synthetic GOX gene with the addition of CTP 1 was synthesised with information 20 from patent publication WO92/00377 with addition of *Nco*I site at the translation start ATG, and a *Kpn* I at the 5' end. Internal *Sph* I sites and *Nco*I site were deleted during synthesis with no change in amino acid usage. The CTP 1 GOX synthesised sequence was isolated as a *Nco* I *Kpn* I fragment and ligated using standard molecular cloning techniques into *Nco*I *Kpn*I cut pMJB1, a plasmid based on pIBT 211 containing the CaMV 35 promoter with duplicated 25 enhancer linked to the tobacco mosaic virus translational enhancer sequence replacing the tobacco etch virus 5' non-translated leader, and terminated with the nopaline synthase poly (A) signal (nos) (Figure 8).

A cassette containing enhanced 35 CaMV TMV sequence CTP1 GOX and nos terminator (dicot vector 1 pUC Figure 17) was isolated as a *Hind*III *Eco*RI fragment and 30 ligated into *Hind* III *Eco*RI cut pJRII, a Bin 19 base plant transformation vector (Figure 9).

Dicot Vector 2

The synthetic EPSPS CP4 gene, fused to the chloroplast transit sequence CTP2 (Figure 10) from EPSPS class I gene from Petunia hybrida, was synthesised with data from patent WO 92/04449 with *Nco*I at the translation initiation ATG. A internal *Sph* I site was 5 silenced in the EPSPS CP4 gene with no change of amino acid usage.

A fragment containing the synthetic CTP 2 CP4 EPSPS was isolated as a *Nco*I *Sac* I fragment and ligated in to pMJBI. A fragment containing the CaMV 35 promoter with a duplicated enhancer, TMV omega sequence CTP 2 transit peptide, EPSPS and nos terminator was isolated as a *Eco*RI *Hind* III fragment (dicot vector 2 pUC Figure 18) and cloned into 10 pJRIi to give dicot vector 2 pUC (Figure 18).

Upon sequencing the junctions of dicot vector 2, an additional sequence was identified inserted between the *Sac*I site and the beginning of the nos terminator. This was as follows:

5' AGG CTG CTT GAT GAG CTC GGT ACC CGG GGA TCC ATG GAG CCG AAT 3'

15

Dicot Vector 3

A control vector with both EPSPS and GOX genes was constructed by cutting dicot vector 2 with *Eco*RI and inserting an  $\Delta$ *Eco*RI *Sph* I  $\Delta$ *Eco*RI linker. The sequence of the linker is shown below:

20

5' AAT TAG GGG CAT GCC CCT 3'

The resultant vector was cut with *Sph* I to liberate the cassette B which was cloned into an *Sph*I site in dicot vector 1), 5' to the 35 CaMV promoter. Cassettes 1) and 2) were then excised as a *Hind*III and *Eco*RI fragment from dicot vector 3- pUC (Figure 19) and cloned in to pJRIi.

25

Dicot Vector 4

An inducible GOX vector was constructed by excising the CAT gene from "p<sub>alc</sub>CAT" as *Pst*I fragment. The vector band, containing the *alcA* promoter and nos terminator was gel purified and used in ligations with a *Pst*I-*Xho*I-*Kpn*I-*Pst*I linker, the sequence of which is as follows:

30

5' GCC ACT CGA GCT AGG TAC CCT GCA 3'

The orientation of this was confirmed by sequence analysis. The TMV omega and CTP1 GOX sequence from dicot vector 1) were isolated as a *Xho*I *Kpn*I fragment and cloned into the *alcA* nos vector containing the *Xho*I-*Kpn*I-*Pst*I linker. The *alcA* TMV CTP1 GOX

nos cassette was excised as a *Hind*III fragment and cloned into the plant transformation vector "p35S-*alc* R", containing the *alc*R cDNA nos terminator under the control of the 35 CaMV promoter to form dicot vector 4 (Figure 20).

#### Dicot Vector 5

5 Dicot vector 5 (Figure 22) containing inducible GOX and constitutive EPSPS genes was prepared using the following cloning strategy. Dicot vector 2 (pDV2 -pUC) was modified by cloning in a  $\Delta$ *Eco*RI-*Hind*III- $\Delta$ *Eco*RI linker into the *Eco*RI site to allow excision of the CaMV en-CTP2-EPSPS -nos cassette as a *Hind*III fragment. This fragment was then ligated into *Hind*III cut pDV4-Bin. Recombinants containing all three cassettes ie 35S-*Alc*R, 10 CaMV-en-CTP2-EPSPS-nos and *Alc*A-CTP1-GOX-nos were selected by hybridization with radiolabelled oligonucleotides. Confirmation of orientation was done by sequencing across all borders.

#### Monocot Vectors

##### **Vector 1: Cassette D**

15 An *Eco*RI-*Not*I-*Eco*RI linker (5'AATTCA $\Delta$ TTGC $\Delta$ GGCCGCAAATG3') was inserted into dicot vector pDV1. The plasmid was cut with *Nco*I and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *Not*I and the resulting blunt/*Not*I fragment containing the CTP1 GOX and nos terminator was ligated into a *Sma*I/*Not*I digested pPUB1 vector (Figure 12) containing the polyubiquitin promoter, 20 polyubiquitin intron with a *Kpn*I-*Not*I-*Kpn*I linker (5'CATTGCGGCCGC AAATGGTAC3') insertion. A *Hind*III-*Not*I-*Hind*III linker (5'AGCTTGAGCGGC CGCTGCA3') was inserted into the resulting construct.

##### **Vector 1: Cassette E**

25 An *Eco*RI-*Not*I-*Eco*RI linker (5'AATTCA $\Delta$ TTGC $\Delta$ GGCCGCAAATG3') was inserted into dicot vector pDV2. The plasmid was cut with *Nco*I and the 5' overhang filled-in with DNA Polymerase I Klenow fragment. The linear vector was then cut with *Not*I and the resulting blunt/*Not*I fragment containing the CTP2 EPSPS and nos terminator was ligated into a *Sma*I/*Not*I digested pPUB1 vector containing the polyubiquitin promoter, polyubiquitin intron 30 with a *Kpn*I-*Not*I-*Kpn*I linker (5'CATTGCGGCCGCAAATGGT AC3') insertion to create plasmid 1. The PAT selectable marker cassette (35S CaMV promoter, *Adh*I intron,

phosphinothricin acetyl transferase gene (PAT), nos terminator) was excised from pIE108 (Figure 14) and cloned into the *Hind*III site on plasmid 1 to give monocot cassette E. Diagnostic restriction digestion was used to confirm that the selectable marker cassette was inserted 5' to 3' in the same orientation as the CTP2 EPSPS cassette.

5 A fragment containing the polyubiquitin promoter, polyubiquitin intron, CTP1 GOX and nos terminator was excised from cassette D with *Not*I and ligated into *Not*I cassette E to form monocot vector 1 (Figure 14). Restriction digestion was used to confirm that the two cassettes were inserted in the same orientation.

10 The selectable marker cassette (35 CaMV promoter, *Adh*I intron, phosphinothricin acetyl transferase gene (PAT), nos) was excised from pIE108 and cloned into the *Hind* III site in 5) to give monocot cassette E.

#### Vector 1

15 A fragment containing the polyubiquitin promoter, polyubiquitin intron GOX and nos was excised from cassette D with *Not*I and cloned into *Not*I cut cassette E, to form monocot vector 1.

#### Vector 2 Cassette F

An *Eco*RI fragment from pUC4 (Figure 15) containing the *alcR* cDNA and nos terminator sequences was blunt end-filled with DNA Polymerase I Klenow fragment, ligated into pUB1 with the *Kpn*I-*Not*I-*Kpn*I linker insertion and orientated by restriction analysis. The PAT selectable marker cassette was inserted in the *Hind*III site after excision from pIE108 and orientated by restriction analysis to create vector 1. Plasmid 1 above containing the polyubiquitin promoter, polyubiquitin intron, CTP2 EPSPS and nos terminator was cut with *Hind*III and a  $\Delta$ *Hind*III-*Not*I-*Hind*III linker:

25 5' AGCTCGCAGCGGCCGCTGCA3'

5' GCGTCGCCGGCAGCTTCGA3'

inserted and orientated by sequencing to create vector 2.

30 A *Cl*I-*Nco*I-*Cl*I linker (5'CGATGCAGCCATGGCTGCAT3') was inserted into pMF6 (Figure 13) to give vector 3. An *Nco*I/*Kpn*I fragment containing CTP1 GOX was excised from pDV1 and inserted into *Nco*I/*Kpn*I cut vector 3 to create vector 4. A *Sal*I fragment containing the maize *Adh*I intron, CTP1 GOX was excised from vector 4 and ligated into *Sal*I cut pUC2 containing the *alcA* promoter and nos terminator and orientated by sequencing to create vector 5. A *Hind*III fragment from vector 5 containing the *alcA* promoter, maize *Adh*I

intron, CTP1 GOX and nos terminator was ligated into *Hind*III cut vector 2 and orientated by restriction digestion. A *Not*I fragment from the resulting construct containing polyubiquitin promoter, polyubiquitin intron; CTP2 EPSPS, nos terminator, *alcA* promoter, maize *Adh*I intron, CTP1 GOX and nos terminator was ligated into *Not*I cut vector 1 and orientated by restriction analysis to create monocot vector 2 (Figure 16).

#### EXAMPLE 4

##### Plant Transformation

10 Plasmids for dicot transformation were transferred to *Agrobacterium tumefaciens* LBA4404 using the freeze thaw method described by Holsters *et al* 1978.

Tobacco transformants were produced by the leaf disc method described by Bevan 1984. Shoots were regenerated on a medium containing 100 mg/l kanamycin. After rooting plants were transferred to the glasshouse and grown under 16h light/8h dark conditions.

15 Oilseed rape (*Brassica napus* cv westar) transformations were performed using the cotyledon petiole method described by Moloney *et al* 1989. Selection of transformed material was performed on kanamycin (15 mg/l). Rooted shoots were transferred directly to a soil based compost and grown to maturity under controlled glasshouse conditions (16h day 20°C day, 15°C night 60% RH).

20 Maize transformation was performed using the particle bombardment approach as described by Klein *et al* 1988. Selections were performed on 1 mg/l biolophos.

Sugar beet transformation was performed using the guard cell protoplast procedure see our International Patent Publication No. WO95/10178.

Results showing details of the transgenic plants obtained are shown in Tables 2 and 3 below.

25

Table 2 - Transformation Details For Tobacco

Vector	Species	Shoots removed	Rooted
pDV1	Tobacco	150	57
pDV2	Tobacco	150	60
pDV3	Tobacco	270	77
30 pDV4	Tobacco	350	135
pDV5	Tobacco	150	75

Table 3 - Transformation Details in Oil Seed Rape

Vector	Species	Shooting Calli	Rooted
pDV1	OSR	14	shoots from 14
pDV2	OSR	13	shoots from 13
5 pDV3	OSR	18	shoots from 18
pDV4	OSR	20	shoots from 20
pDV5	OSR	19	shoots from 18

EXAMPLE 510 **Transgenic Plant Analysis**Polymerase Chain Reaction (PCR)

Genomic DNA for PCR analysis of transgenic plants was prepared according to the method described by Edwards *et al* 1992. PCR was performed using conditions described by Jepson *et al*, Plant Molecular Biology Reporter, 9(2), 131-138 (1991). Primer sets were 15 designed for each of the introduced cassettes.

The plants were analysed using the following oligonucleotide combinations:-

pDV1	TMV1 + GOX1,	GOX3 + nos1
pDV2	TMV1 + EPSPS1,	EPSPS3 + nos1
pDV3	EPSPS3 + GOX1	
20 pDV4	35S + AlcR1,	AlcA2 + GOX1
pDV5	35S + AlcR1,	AlcA2 + GOX1, TMV1 + EPSPS1

Oligonucleotide sequences are given below:-

TMV1	5' CTCGAGTATTTTACAACAATTACCAAC
25 GOX1	5' AATCAAGGTAAACCTTGAATCCA
GOX3	5' ACCACCAACGGTGGTCTGCTGTTGA
NOS1	5' GCATTACATGTTAATTATTACATGCTT
EPSPS1	5' GTGATACGAGTTCACCGCTAGCGAGAC
EPSPS3	5' TACCTTGCCTGGACCAAAGACTCC
30 35S	5' GTCAACATGGTGGAGCAGC
AlcR1	5' GTGAGAGTTATGACTGGAGGCGCATC
AlcA2	5' GTCCGCACGGAGAGCCACAAACGA

Selection on Glyphosate**Kill Curves for Tobacco var Samsun and Brassica napus var Westar on glyphosate**

Both species were tested on a range of glyphosate concentrations by inserting, in the case of tobacco a 5-6mm stem segment carrying a leaf node and in the case of oil seed rape 5 the growing tip plus two leaves into MS medium containing glyphosate at 0, 0.0055, 0.011, 0.0275, 0.055 and 0.01 mM glyphosate isopropylamine salt. The results were scored after two weeks growth as and are given in Table 4 below.

Table 4

10	Conc	Westar	Tobacco
0		Good stem growth, 4-5 new leaves, roots up to 5cm	As OSR
0.005		No stem growth, 1 new leaf, roots to 1cm	No growth in any organ
0.011		No stem growth, no new leaves, roots~0.5cm	"
15	0.0275	No stem growth, no new leaves, roots~2mm	"
	0.055	No growth in any organ, ends of stem blackened	"
	0.01	As for 0.055mM	"

Selection for glyphosate tolerant transformants was performed on glyphosate 20 concentrations of 0.01 and 0.05mM.

Constitutively tolerant plants

Following from the data obtained on wild type plants, pDV1,2 and 3 PCR +ve primary transformants were screened on MS medium containing glyphosate at the levels described 25 above. For tobacco this was done by inserting three or four stem sections per transformant into the medium and using untransformed Samsun as control. Scoring was based on the behaviour of the majority. Plants showing tolerance at the higher concentration of herbicide were grown on to maturity in the glass house, for seed collection.

Segregation Test

30 Seeds were sterilized in 10% bleach for 10 min. After several washes in sterile water 200 seeds were sown on 1/2 MS medium (2.3 g/l MS salt, 1.5% sucrose, 0.8% Bactoagar,

pH 5.9) containing 100 mg/l kanamycin. Seeds were grown at 26°C with 16 hours/8 hours light/dark prior to scoring.

#### Western Analysis

#### **Antibody Generation**

5 GOX and EPSPS protein were over expressed in E.Coli using a pET expression system. Following IPTG induction GOX and EPSPS were electro eluted from the shake flask grown cell paste and used to immunise rabbits (two animals per clone).

#### **Preparation of Tissue Extracts for Immunoblotting**

10 120 mg of leaf tissue plus 60 mg PVPP and 500 µl extraction buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 0.3 mM DTT) were ground with a blender for several minutes. After homogenisation the extract was centrifuged at 15,000 rpm for 15 min. The supernatant was stored at -80° C until required. Protein concentrations in the extract were measured according to Bradford.

#### **SDS-PAGE and Immunoblotting**

15 25 µg protein were separated by SDS-PAGE. The running buffer was 14.4 % (w/v) glycine, 1 % (w/v) SDS and 3 % (w/v) Tris Base. The samples were loaded according to Laemmli.

After SDS-PAGE proteins were electroblotted overnight with 40 mA onto nitrocellulose (Hybond™ C, Amersham) using an electroblot unit from Biorad. The membrane was stained in 0.05 % CPTS dissolved in 12 mM HCl. Blots were rinsed in 12 mM HCl and destained for 5 - 10 20 min in 0.5 M NaHCO<sub>3</sub>, followed by an intensive rinse with H<sub>2</sub>O. Membranes were then blocked, immunodetected and washed according to the Amersham ECL kit. Indirect immunodetections were performed with a 1:10000 dilution of a rabbit anti-GOX or anti-EPSPS polyclonal as first antibody and with a 1:1000 dilution of an anti-rabbit second antibody, associated with horseradish peroxidase. An additional wash was carried out overnight to eliminate background. Detection was 25 performed using the ECL kit from Amersham and the results are shown in Figure 21 in which Lane (1) is the control and the remaining lanes are transformants. The western analysis demonstrates that some transformants are capable of expressing GOX and EPSPS.

#### **Constitutively tolerant plants**

30 Cell extracts were prepared from each glyphosate tolerant plant and the amount of expressed protein estimated by western analysis using antibody appropriate to the

transformant. Plants expressing very high levels of GOX or EPSPS were tested on higher levels of glyphosate to relate level of expression to herbicide tolerance.

#### Inducibly tolerant plants

To demonstrate inducible tolerance to glyphosate PCR positive primary transformants 5 from the transformations with pDV4 and 5 were transferred directly to the glass house. After two weeks the plants were induced by an ethanol root drench (5% solution) and left for 24 hours prior to western analysis performed to assess level of expression of GOX after induction. After a period of time to allow the plants to return to the uninduced state, the western analysis was repeated to allow selection of inducibly tolerant plants. Plants which 10 showed the highest levels of GOX expression following ethanol treatment were taken forward to time course analysis. GOX levels were assessed at 6, 12, 18, 24, 36, 48 hours following ethanol treatment, by western analysis.

High expressing GOX plants for both pDV4 and pDV5 were used in glass house trials to demonstrate inducible glyphosate tolerance. Plants were induced using a range of ethanol 15 concentrations (1-15%) by root drench application to pot grown plants. Following GOX induction plants were sprayed with glyphosate. Wild type controls and uninduced plants were also treated with herbicide.

#### Northern Analysis

Primary transformants containing dicots vector 2), 3), and 5). were analysed by 20 northern blot analysis - using a CTP2 EPSPS probe as a *NcoI Sac I* fragment. Primary transformants containing the dicot vectors 1). 3). were analysed by northern blotting using a CTPI GOX probe as a *NcoI KpnI* fragment. Similarly, transgenic corn lines containing monocot vectors 1). and 2). were analysed using a CTP2 EPSPS probe.

Transformants containing dicot vector 5). or monocot vector 2). were treated with a 25 foliar application of 5% ethanol to induce GOX levels. RNA was isolated 24 hours after treatment and subjected to northern analysis with a CTPI GOX probe.

Primary transformants which were PCR positive for the appropriate cassettes and showed GOX or EPSPS transcript levels were taken for further analysis.

#### Glyphosate Oxidoreductase Assay

30 Assays for glyphosate oxidoreductase were carried out as described by Kishore and Barry (WO 92/00377). These entailed measuring glyphosate - dependent uptake of oxygen

using an oxygen electrode, detection of glyoxylate formation by reaction with 2, 4 - dinitrophenylhydrazine and determination of the hydrozone using HPLC or, preferably, using [3-  $^{14}\text{C}$ ] - glyphosate as the substrate and detecting the formation of radioactive aminomethyl phosphonic acid via HPLC on an anion exchange column.

5 EPSPS Assay

Assays for 5-enol-pyruvylshikimate-3-phosphate (EPSP) synthase activity in plant extracts were carried out (1) by following the disappearance of the phosphoenol pyruvate substrate (as described by Rubin, J.L., Gaines, C.G and Jensen, R.A., in Plant Physiol (1984 75, 839-845) or (2) by conducting the assay in the reverse direction and coupling to pyruvate 10 kinase and lactate dehydrogenase (as described by Mousdale D.M. and Coggins J.R. in Planta (1984) 160, 78-83) or (3) by using 14(-labelled phosphoenol pyruvate as substrate and detecting the formation of radioactive EPSP by HPLC on an anion exchange column and detecting using a flow-through radioactivity detector as described by Della-Cioppa *et al* in Proc. Nat. Acad. Sci. (USA) (1986), 83, 6873-6877. The latter assay was used to confirm 15 that the EPSP synthase activity was, as expected, relatively resistant to inhibition by glyphosate.

CLAIMS

1. A chemically inducible plant gene expression cassette comprising an inducible promoter operatively linked to a target gene which confers resistance to a herbicide.  
5
2. A chemically inducible plant gene expression cassette as claimed in claim 1, wherein the herbicide is N-phosphonomethyl-glycine or a salt or derivative thereof.
3. A chemically inducible plant gene expression cassette as claimed in claim 1 or claim 2, 10 wherein the inducible promoter is the tetracycline repressor gene switch, the Lac repressor system, a copper inducible systems such as that based on ACE 1, a salicylic acid inducible promoters, for example the PR-1a system, a system based on a steroid hormone such as the glucocorticoid, progesterone and oestrogen receptor systems or a modification of one of these such as a glucocorticoid receptor system which includes 15 the GAL 4 binding domain from yeast and the VP16 activator, an insect steroid hormones systems such as that based on the ecdysone receptor of *Heliothis virescens*, a benzene sulphonamide gene switching system, a gene switching based on the *alcR* protein from *Aspergillus nidulans* or a glutathione S-transferase promoter.
- 20 4. A chemically inducible plant gene expression cassette comprising a first promoter operatively linked to an *alcR* regulator sequence which encodes an *alcR* regulator protein, and an inducible promoter operatively linked to a target gene which confers herbicide resistance, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer 25 causes expression of the target gene.
5. A plant gene expression cassette according to claim 4, wherein the inducible promoter is derived from the *alcA*, *alcR*, *aldA* or other *alcR*-induced gene promoter.
- 30 6. A plant gene expression cassette according to either of claims 4 and 5, wherein the inducible promoter is a chimeric promoter.

7. A plant gene expression cassette according to any preceding claim, wherein the target gene confers resistance to the herbicide N-phosphonomethyl-glycine or a salt or derivative thereof.
- 5
8. A plant cell containing a plant gene expression cassette according to any preceding claim.
9. A plant cell according to claim 8, wherein the plant gene expression cassette is stably incorporated in the plant's genome.
- 10
10. A plant tissue comprising a plant cell according to either of claims 8 and 9.
11. A plant comprising a plant cell according to either of claims 8 and 9.
- 15
12. A plant derived from a plant according to claim 11.
13. A seed derived from a plant according to either of claims 11 and 12.
- 20
14. A method of controlling herbicide resistance comprising transforming a plant cell with the plant gene expression cassette of any one of claims 1 to 7.
15. A method of selectively controlling weeds in a field of plants according to either of claims 11 or 12, or seeds according to claim 13, comprising applying an effective amount of the herbicide and the exogenous inducer.
- 25

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Fig.2.



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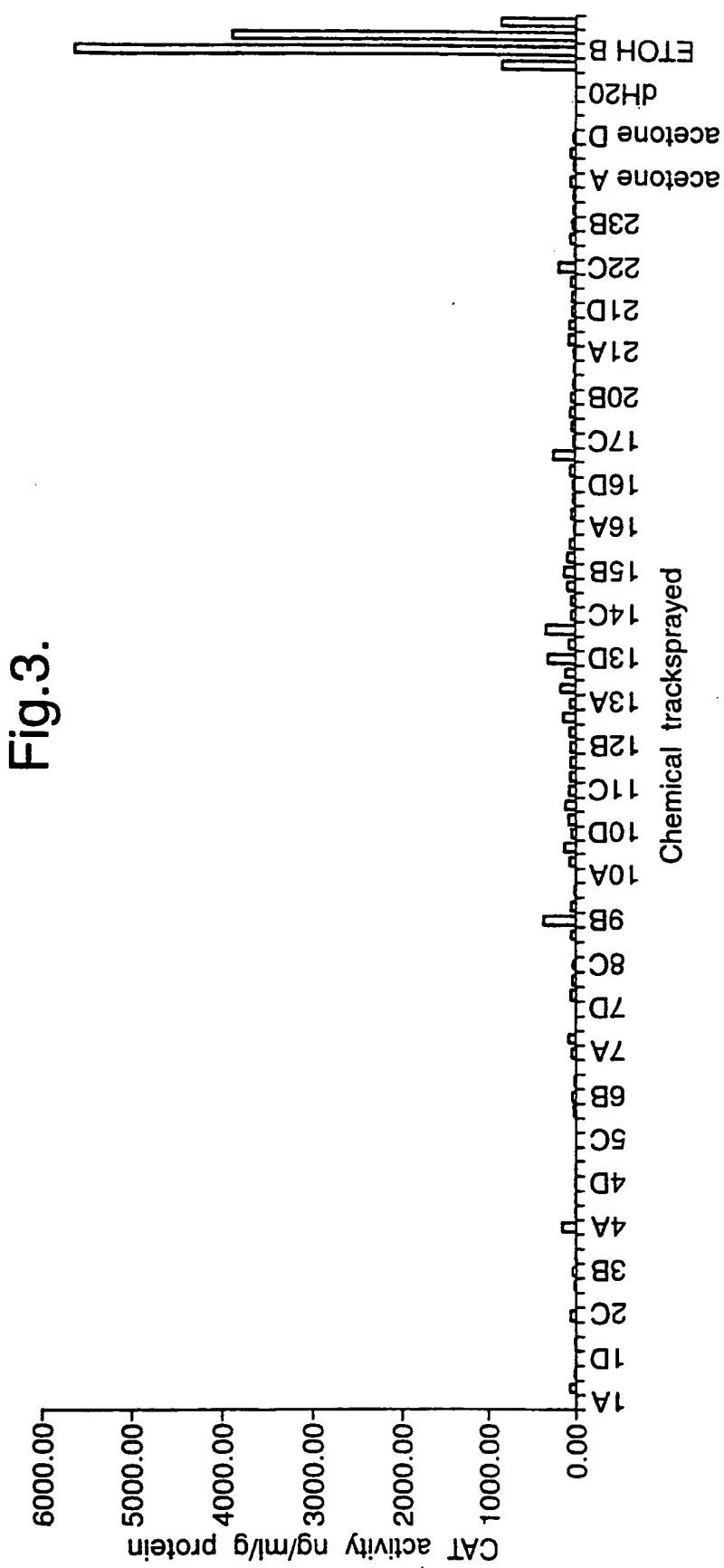


Fig.3.

Fig. 4(1/2).

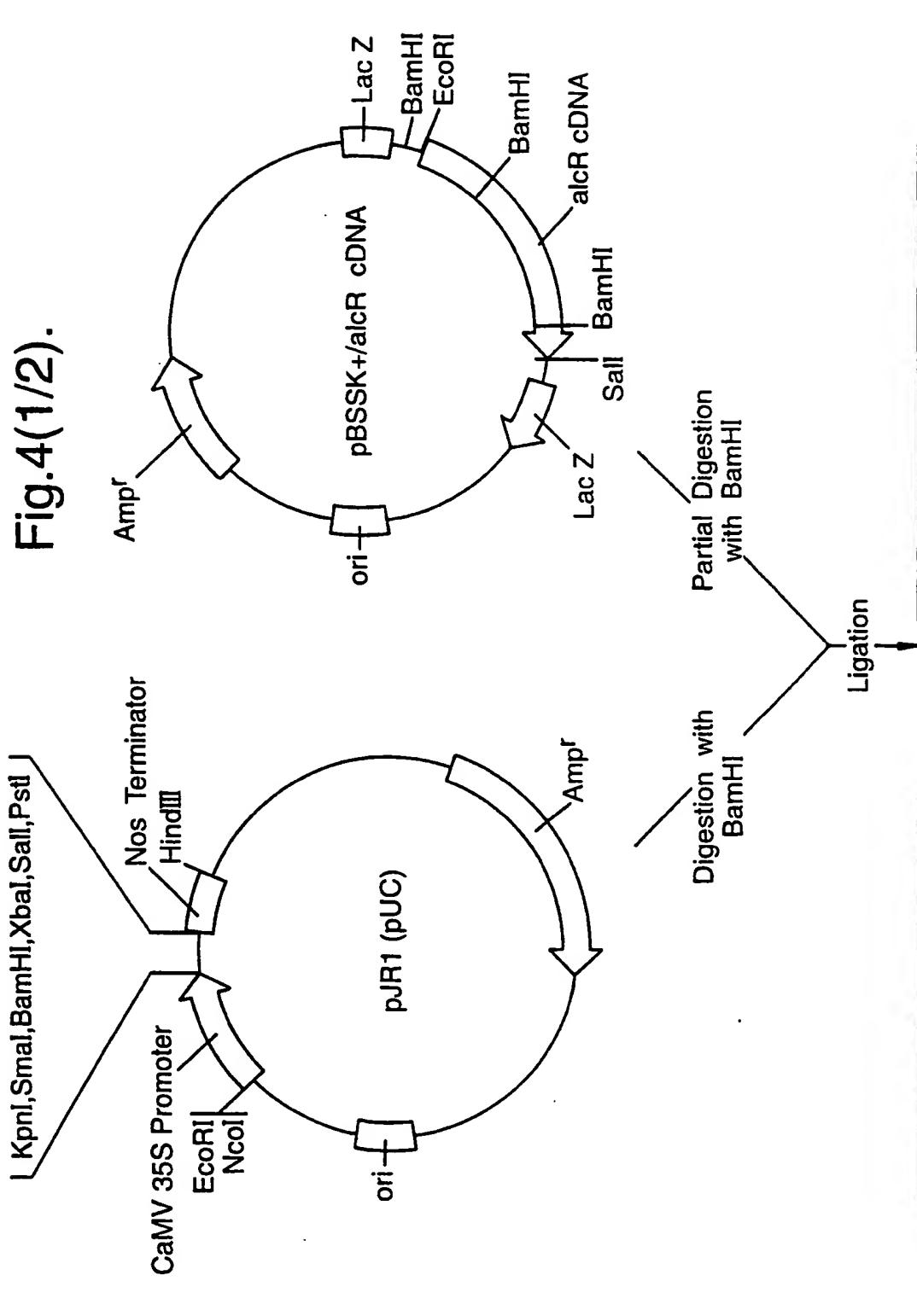
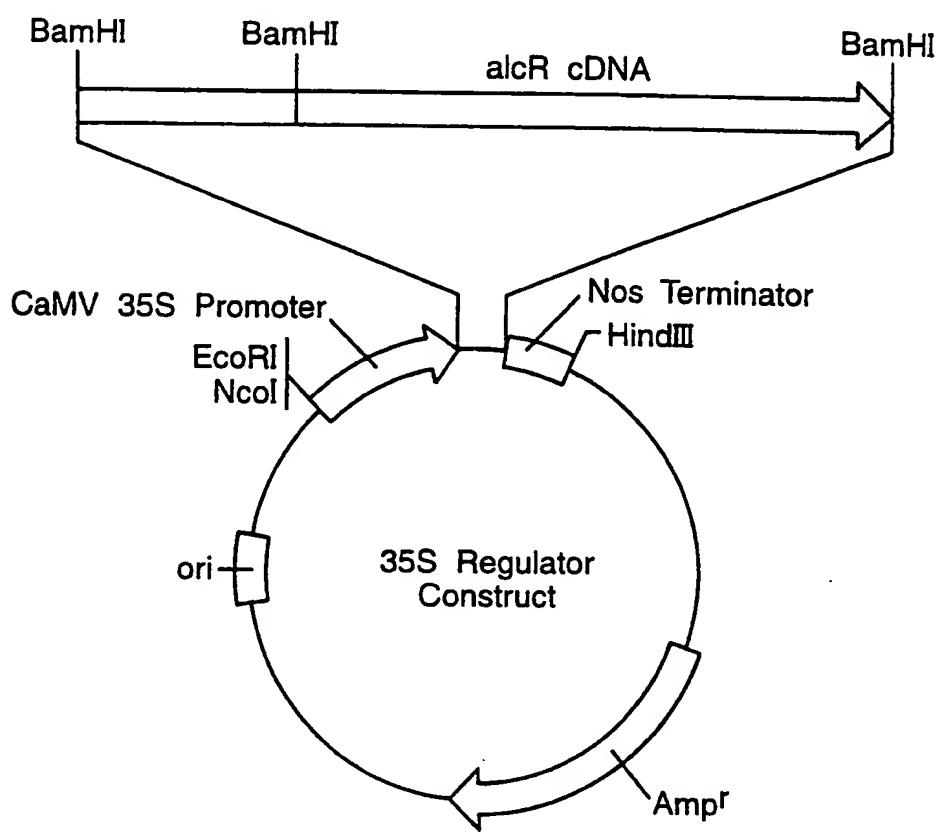
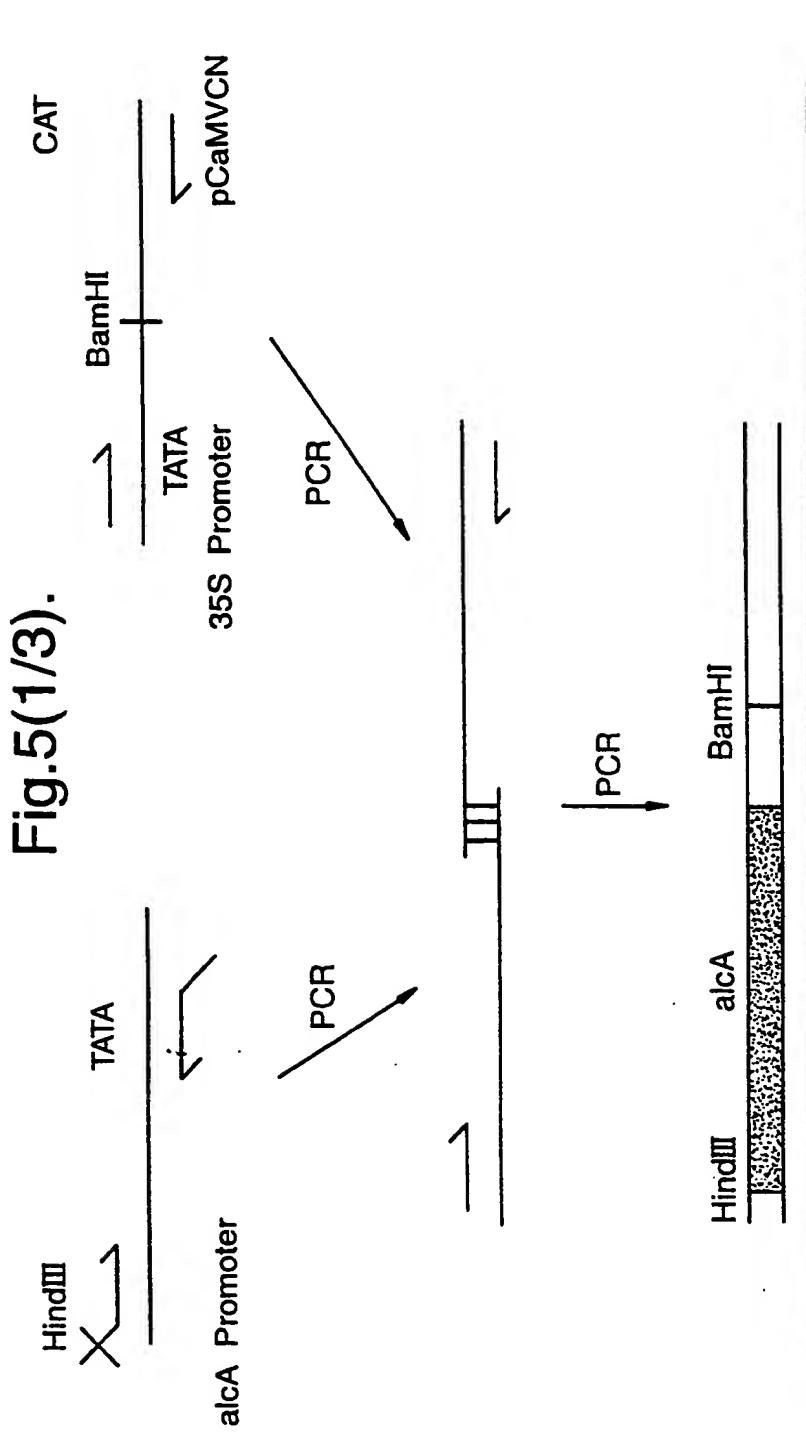


Fig.4(2/2).





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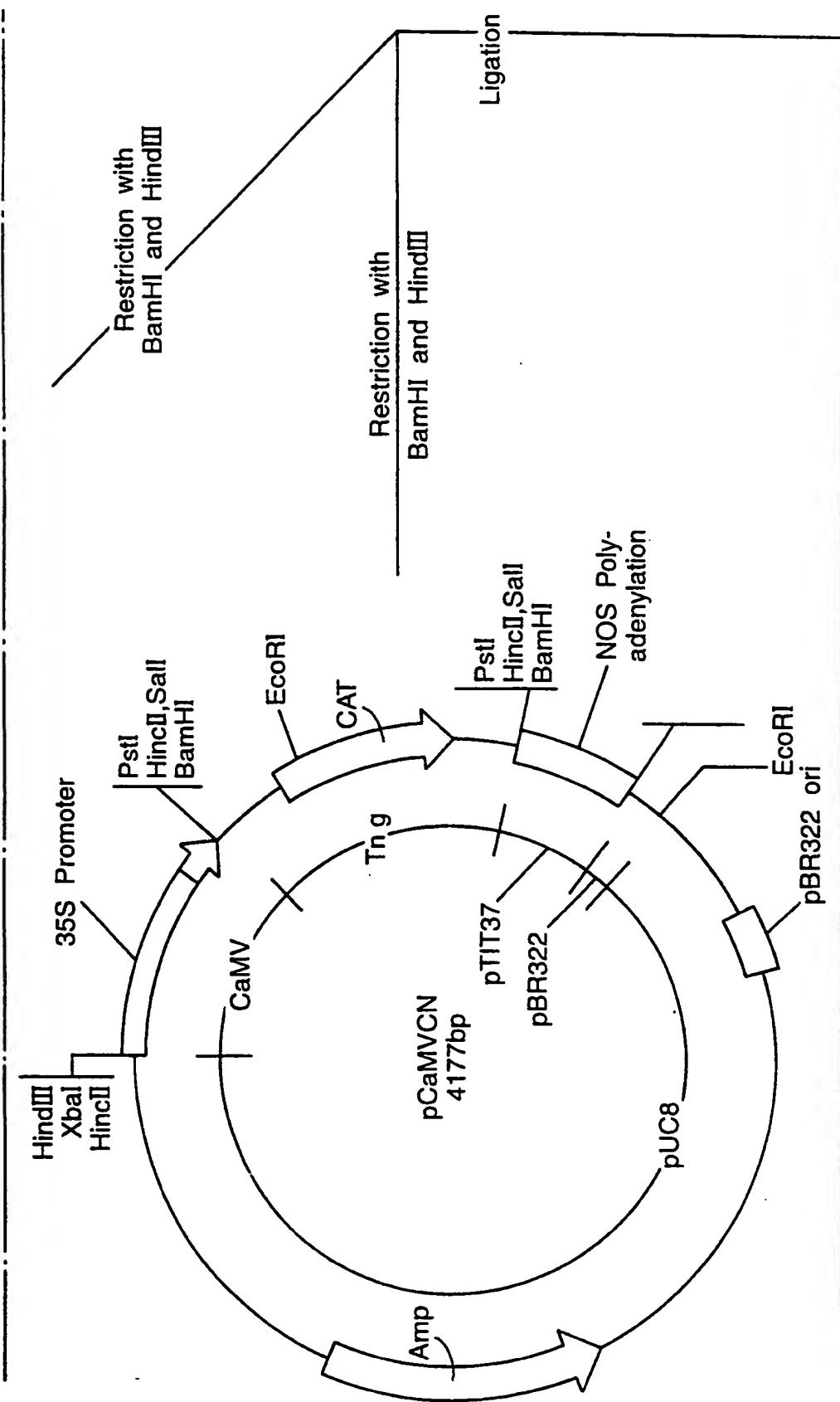
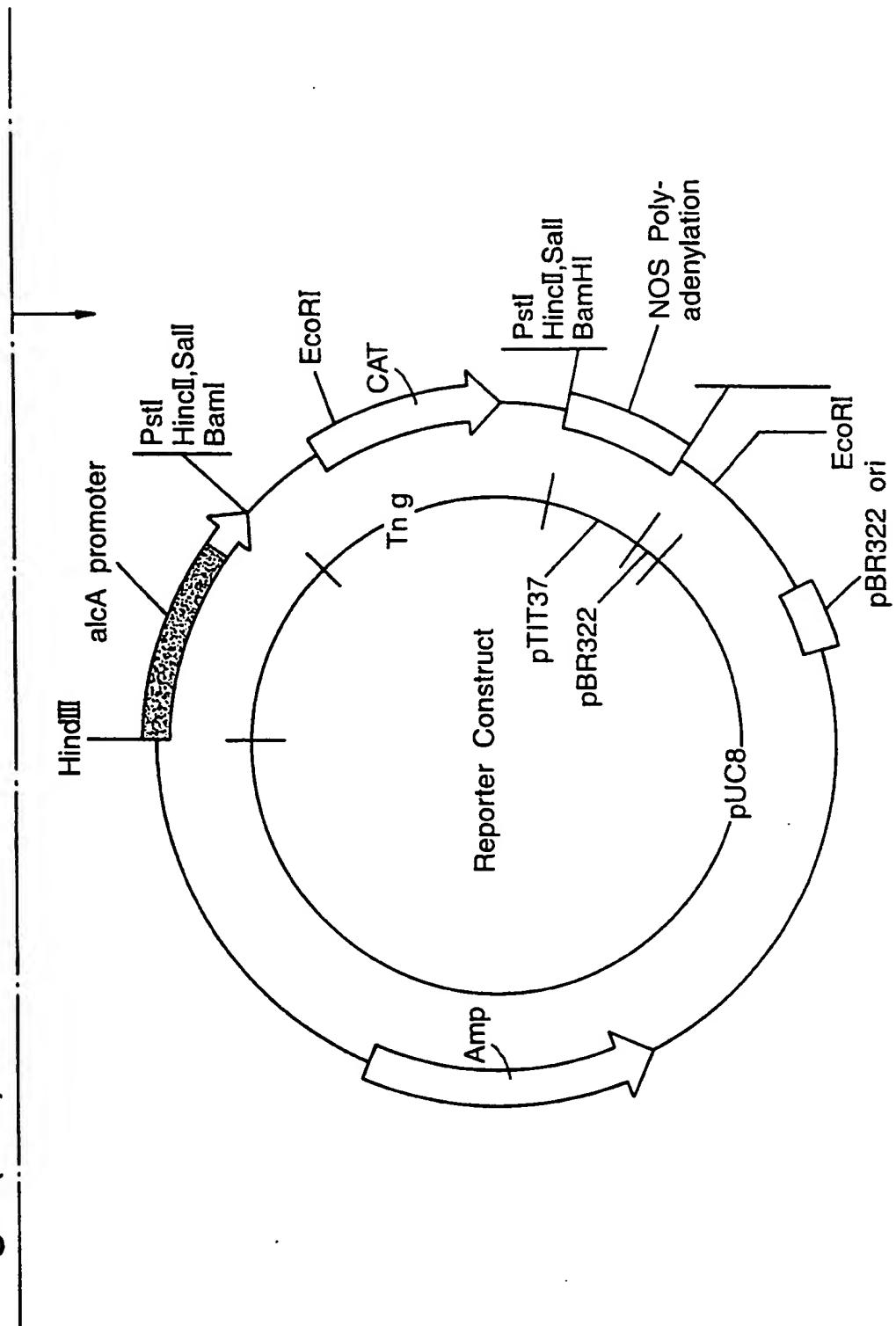
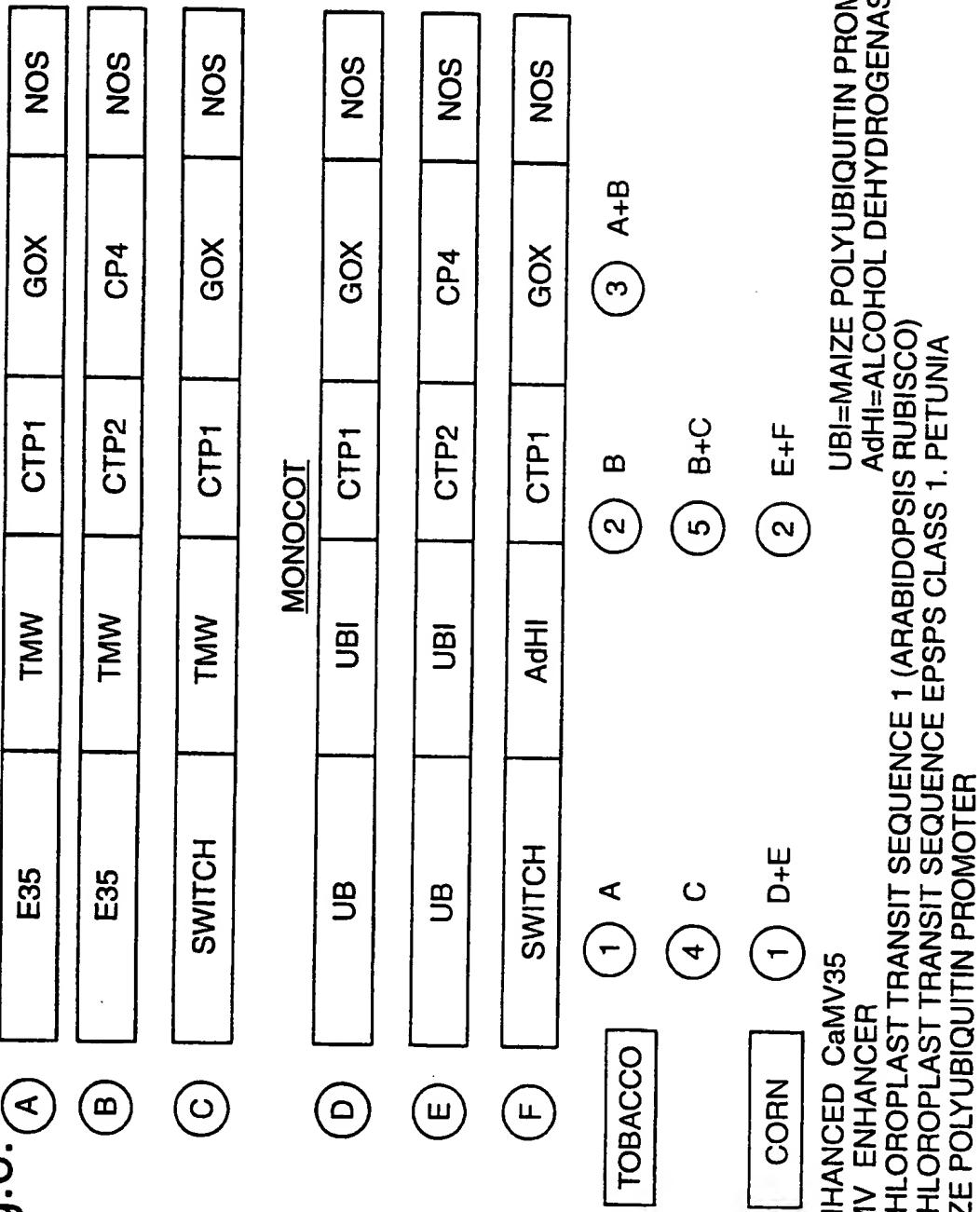
**Fig.5(2/3).**

Fig.5(3/3).



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## DICOT



## **SUBSTITUTE SHEET (RULE 26)**

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Fig.7(1/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACCA	TGGCTTCCTC	TATGCTCTCT	TCCGCTACTA	TGGTTCCTC	50
K L T M	A S S	M L S	S A T M	V A S	
TCCGGCTCAG	GCCACTATGG	TCGCTCCCTT	CAACGGACTT	AAGTCCTCCG	100
P A Q	A T M V	A P F	N G L	K S S A	
CTGCCTTCCC	AGCCACCCGC	AAGGCTAACAA	ACGACATTAC	TTCCATCACA	150
A F P	A T R	K A N N	D I T	S I T	
AGCAACGGCG	GAAGAGTTAA	CTGTATGCAG	GTGTGGCTC	CGATTGGAAA	200
S N G G	R V N	C M Q	V W P P	I G K	
GAAGAAGTTT	GAGACTCTCT	CTTACCTTCC	TGACCTTACC	GATTCCGGTG	250
K K F	E T L S	Y L P	D L T	D S G G	
GTCGCGTCAA	CTGTATGCAG	GCTATGGCTG	AGAACACAA	GAAGGTGGT	300
R V N	C M Q	A M A E	N H K	K V G	
ATCGCTGGAG	CTGGAATCGT	TGGTGTTCGC	ACTGCCTTGA	TGCTTCAACG	350
I A G A	G I V	G V C	T A L M	L Q R	
TCGTGGATTIC	AAGGTTACCT	TGATTGATCC	AAACCCACCA	GGTGAAGGTG	400
R G F	K V T L	I D P	N P P	G E G A	
CTCTTTCCG	TAACGCTGGT	TGCTTCAACG	GTTCCTCCGT	TGTTCCAATG	450
S F G	N A G	C F N G	S S V	V P M	
TCCATGCCAG	GAAACTTGAC	TAGCGTTCCA	AAGTGGCTTC	TGGATCCTGT	500
S M P G	N L T	S V P	K W L L	D P V	
TGTGAATTIC					509
V N					

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## Fig.7(2/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTTACGG	ATCCAATGGG	TCCATTGTCC	ATCCGTTTCA	GCTACTTTCC	50
K L T D	P M G	P L S	I R F S	Y F P	
AACCATCATG	CCITGGITGA	TTCGTTCTT	GCTTGCTGGA	AGACCAAACA	100
T I M	P W L I	R F L	L A G	R P N K	
AGGTGAAGGA	GCAAGCTAAG	GCACTCCGTA	ACCTCATCAA	GTCCACTGTG	150
V K E	Q A K	A L R N	L I K	S T V	
CCTTGATCA	AGTCCTTGGC	TGAGGAGGCT	GATGCTAGCC	ACCTTATCCG	200
P L I K	S L A	E E A	D A S H	L I R	
TCACGAAGGT	CACCTTACCG	TGTACCGTGG	AGAAGCAGAC	TTCGCCAAGG	250
H E G	H L T V	Y R G	E A D	F A K D	
ACCGTGGAGG	TTGGGAACCT	CGTCGTCTCA	ACGGTGTTCG	TACTCAAATC	300
R G G	W E L	R R L N	G V R	T Q I	
CTCAGCGCTG	ATGCATTGCG	TGATTTCGAT	CCTAACTTGT	CTCACGCCCT	350
L S A D	A L R	D F D	P N L S	H A F	
TACCAAGGGA	ATCCTTATCG	AAGAGAACGG	TCACACCATC	AACCCACAAG	400
T K G	I L I E	E N G	H T I	N P Q G	
GTCTCGTAC	TCTCTTGTCT	CGTCGTCTCA	TCGCTAACGG	TGGAGAGTTC	450
L V T	L L F	R R F I	A N G	G E F	
GIGTCTGCCTC	GIGTATCGG	ATTGGAGACT	GAAGGGTCTG	CCTCTCAAGGG	500
V S A R	V I G	F E T	E G R A	L K G	
TATCACCACC	ACCAACGGTG	TTCTTGCTGT	TGAATGCTGCA	GIGTGTGAA	550
I T T	T N G V	L A V	D A A	V L . I	
TTC					553

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## Fig.7(3/3).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	1234567890
AAGCTTACTG	CAGITGTTGC	AGCTGGTGCA	CACTCCAAGT	CTCTTGCTAA	50
K L T A	V V A	A G A	H S K S	L A N	
CTCCCTTGGT	GATGACATCC	CATTGGATAC	CGAACGTGGA	TACCACATCG	100
S L G	D D I P	L D T	E R G	Y H I V	
TGATGCCAA	CCCAGAAGCT	GCTCCACGTA	TICCAACTAC	CGATGCCCT	150
I A N	P E A	A P R I	P T T	D A S	
GGAAAGTTCA	TCGCTACTCC	TATGGAGATG	GGTCTTCGIG	TTGCTGGAAC	200
G K F I	A T P	M E M	G L R V	A G T	
CGTTGAGTTC	GCTGGCTCA	CTGCTGCTCC	TAACCTGGAAG	CGTGCTCACG	250
V E F	A G L T	A A P	N W K	R A H V	
TTCTCTACAC	TCGTGCTCGT	AAGTTGCTTC	CAGCTCTCGC	TCCTGCCAGT	300
L Y T	R A R	K L L P	A L A	P A S	
TCTGAAGAAC	GTTACTCCAA	GTGGATGGGT	TTCCGTCCAA	GCATCCCAGA	350
S E E R	Y S K	W M G	F R P S	I P D	
TTCCCTTCCA	GTGATTGGTC	GTGCTACCCG	TACTCCAGAC	GTATCTACG	400
S L P	V I G R	A T R	T P D	V I Y A	
CTTTCGGTCA	CGGTACCTC	GGTATGACTG	GTGCTCCAAT	GACCGCAACC	450
F G H	G H L	G M T G	A P M	T A T	
CICGTTCTIG	AGCTCCTCCG	AGGTGAGAAG	ACCTCTATCG	ACATCTCTCC	500
L V S E	L L A	G E K	T S I D	I S P	
ATTCGCACCA	AACCGTTTCG	GTATTGGTAA	GTCCAAGCAA	ACIGGTCTTG	550
F A P	N R F G	I G K	S K Q	T G P A	
CATCCTAAGG	TACCGAATT				570
S . G	T E F				

Fig. 8.

HindIII 10 SphI PsI 20 ↑<sup>35S</sup> 30 40 50 60

1 AAGCTTGCAT GCCTGCAGGT CAACATGGTG GAGCACCACA CACTTGTCTA CTCCAAAAT  
TTCGAACGTT CGAACGTCAC GTTGTACAC CTCGTGCTGT GTGAACAGAT GAGGTTTTA

61 ATCAAAGATA CAGTCTCAGA AGACCAAAGG GCAATTGAGA CTTTCAACA AAGGGTAATA  
TAGTTTCTAT GTCAAGAGTCT TCTGGTTCC CGTTAACCT GTAAAGTTGT TTCCCATAT

121 TCGGAAACC TCCTCGGATT CCATTGGCCA GCTATCTGTC ACCTTATTGT GAAGATAAGTG  
AGGCCCTTGG AGGAGCCTAA GGTAACGGGT CGATAGACAG TGAAATAACA CTTCTATCAC

181 GAAAGGAAG GTGGCTCCTA CAAATGCCAT CATTGGATA AAGGAAAGGC CATCGTTGAA  
CTTTCCCTTC CACCGAGGAT GTTACGGTA GAAACGCTAT TTCCCTTCG GTAGCAACTT

241 GATGCCTCTG CCGACAGTGG TCCCAAAGAT GGACCCCCAC CCACGGAGG CATCGTTGAA  
CTACGGAGAC GGCTGTCACC CCTGGGGGTG GGTGCTCCCTC GTAGCACCTT

DUF ATC EN

Fig. 8 (Cont).

301 AAAAGAAGAC GTTCCAACCA CGTCTCTAAA GCAAGTGGAT TGATGTGATA ACATGGTGGAA  
 361 GCACGACACA CTTGCTACT CCAAAATAAT CAAAGATACA GTCTCAGAAG ACCAAAGGGC  
 CGTGCCTGGT GAAACGATGA GGTTTTATA GTTTCTATGT CAGAGTCTTC TGGTTTCCCG

421 ATTGAGACT TTTCACACAA GGGTAATATC CGGAAACCTC CTCGGATTCC ATTGCCAGC  
 TTAACTCTGA AAAGTTGTTT CCCATATAG GCCTTTGGAG GAGCCTAAGG TAACGGGTGCG

481 TATCTGTCACT TTTATTGTGA AGATAGTGGAA AAAGGAAGGT GGCTCCTACAA AATGCCATCA  
 ATAGACAGTG AAAATACACT TCTATCACCT TTCCCTTCCA CGGAGGATGT TTACGGTAGT

541 TTGGGATAAA GGAAAGGCCA TGTTGAAAGA TGCCTCTGGCC GACAGTGGTC CCAAAGATGG  
 AACGGCTATT CCTTCCGGT AGCAACTTCT ACGGAGACGG CTGTCCACAG GGTTCTACCC

601 ACCCCCCACCC ACGAGGGAGCA TCGTGGAAAA AAGAAGACGT TCCAACCACG TCTTCAAAGC  
 TGGGGTGGG TGCTCCCTGT AGCACCTTT TTCTTCTGCA AGGGTGGTC AGAAGTTTCG  
 EcoRV

661 AAGTGGATGG ATGGTATATC TCCACTGACG TAAGGGATGA CGCACAAATCC CACTATCCTT  
 TTACCTAAC TACACTATAG AGGTGACTTGC ATTCCCTACT GGTTGTTAGG GTGATAGGAA

35S PR

Promoter

Fig. 8 (Cont i).

721 CGCAAGACCC TTCCCTTATA TAGGAAAGT CATTTCATT GGAGGGACCC TCGAGTATT  
GGGTTCTGGG AAGGAGATAT ATTCCCTCAA GTAAAGTAA CCTCTCTGG AGCTCATAAA  
TATA BOX

781 TTACAACCAT TACCAACAC AACAAACAAAC AAACAAACATT ACAATTACTA TTACAAATT  
AATGTTGTTA ATGGTTGTG TGGTTGGTG TTTGTTGTA TGTTAATGAT AATGTTAT  
Ncol SmaI KpnI SacI

841 CACCATGGAT CCCCCGGTAC CGAGCTCGAA TTTCCCGAT CGTTCAAAACA TTGGCAATA  
GGGGCCCATG GCTCGAGCTT AAAGGGCTA GCAAGTTGTA AAACCGTTAT  
BamHI

901 AAGTTTCTTA AGATTGAATC CTGTTGCCGG TCTTGGATG ATTATCATAT AATTCTGTGTT  
TTCAAAAGAT TCTAATCTAG GACAACGGCC AGAACGCTAC TAATAGTATA TTAAAGACAA

961 GAATTACGTT AAGCATGTA TAATTAACAT GTAATGCATG ACGTTATTTA TGAGATGGGT  
CTTAATGCCA TTCTGTACATT ATTAATTGTA CATTACGTAC TGCAATAAAT ACTCTACCCA

1021 TTTTATGATT AGAGTCCCGC AATTATACAT TAAATACGG ATAGAAACAA AAATATAGCG  
AAAATACAA TCTCAGGGCC TAAATATGTA AATTATGCC TATCTTTGT TTATATGCC

1081 CGCAAACTAG GATAAATTAT CGCGCGCGGT GTCACTATG TTACTAGATC GGAATTG  
GGGTTGATC CTATTAATA CGCGCGGCCA CAGTAGATAAC AATGATCTAG CCCTTAAG  
EcoRI

TSP Xhol

Ω

TM

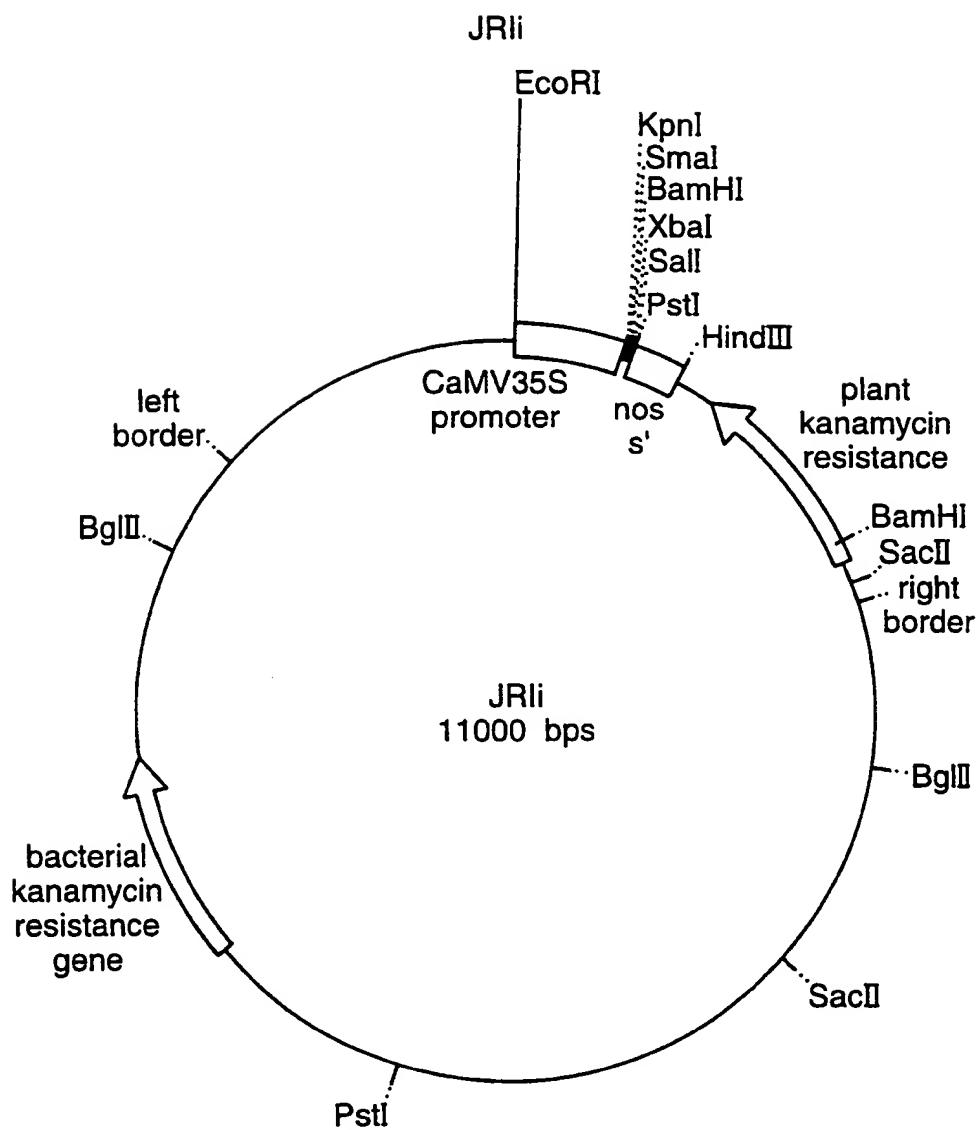
NOS

3'

Total number of bases is: 1138.  
DNA sequence composition: 370 A; 253 C; 234 G; 281 T; 0 OTHER;

Sequence name: PMJB1

Fig.9.



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Fig.10(1/4).

10	20	30	40	50													
<u>1234567890</u>					<u>1234567890</u>												
<u>1234567890</u>					<u>1234567890</u>												
<u>1234567890</u>					<u>1234567890</u>												
<u>1234567890</u>					<u>1234567890</u>												
K	L	T	M	A	Q	V	S	R	I	C	N	G	V	Q	N	P	
ATCTCTTATC TCCAATCTCT CGAAATCCAG TCAACGCAAA TCTCCCTTAT																100	
S	L	I	S	N	L	S	K	S	S	Q	R	K	S	P	L	S	
CGGTTTCTCT GAAGACCGAG CAGCATCCAC GAGCCTATCC GATTTCGTG																150	
V	S	L	K	T	Q	Q	H	P	R	A	Y	P	I	S	S		
TCGTGGGAT TGAAGAAGAG TGGGATGACG TTAATTGGCT CTGAGCTTCG																200	
S	W	G	L	K	K	S	G	M	T	L	I	G	S	E	L	R	
TCCTCTTAAG GTCAATGCTT CTGTTTCCAC GGCGTGTATG CTTCACGGTG																250	
P	L	K	V	M	S	S	V	S	T	A	C	M	L	H	G	A	
CAAGCAGCCG TCCAGCAACT GCTCGTAAGT CCTCTGGTCT TTCTGGAAC																300	
S	S	R	P	A	T	A	R	K	S	S	G	L	S	G	T		
GTCCGTATTG CAGGTGACAA GTCTATCTCC CACAGGTCTT TCATGTTGG																350	
V	R	I	P	G	D	K	S	I	S	H	R	S	F	M	F	G	
AGGTCTCGCT AGCGGTGAAA CTCTATCAC CGGTCTTTCG GAAGGTGAAG																400	
G	L	A	S	G	E	T	R	I	T	G	L	L	E	G	E	D	
ATGTTATCAA CACTGGTAAG GCTATGCAAG CTATGGGTGC CAGGATCCTG																450	
V	I	N	T	G	K	A	M	Q	A	M	G	A	R	I	L		
TGTGAAATTG																460	
L	.	I															

Fig.10(2/4).

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10	20	30	40	50	
<u>1234567890</u>					<u>1234567890</u>
<u>1234567890</u>					<u>1234567890</u>
<u>1234567890</u>					<u>1234567890</u>
<u>1234567890</u>					<u>1234567890</u>
<u>1234567890</u>					<u>1234567890</u>
					50
A	A	G	C	T	
K	L	R	I	R	K
E	L	A	P	K	E
G	A	P	A	E	A
D	T	A	P	P	D
T	D	T	A	A	F
W	I	I	I	I	G
I	D	D	D	D	V
D	G	V	G	G	G
TAACGGTGGA	CTCCTTGCTC	CTGAGGCTCC	TCTCGATTTC	GGTAACGCTG	100
N	G	G	L	L	A
G	G	L	L	A	P
CAACTGGTIG	CCGTTTGACT	ATGGGTCTTG	TTGGTGTTTA	CGATTTCGAT	150
T	G	C	R	L	T
G	C	R	L	T	M
AGCACTTCA	TTGGTGACGC	TTCTCTCACT	AAGCGTCCAA	TGGGTCGTGT	200
S	T	F	I	G	D
T	F	I	G	D	A
GTGAAACCCA	CTTCGCGAAA	TGGGTGTGCA	GGTGAAGTCT	GAAGACGGTG	250
L	N	P	L	R	E
N	P	L	R	E	M
ATCGCTTCC	AGTACCTTG	CGTGGACCAA	AGACTCCAAC	GCCAAATCACC	300
R	L	P	V	T	L
L	P	V	T	L	R
TACAGGGTAC	CTATGGCTTC	CGCTCAAGTG	AAGTCCGCTG	TTCIGCTTGC	350
Y	R	V	P	M	A
R	V	P	M	A	S
TGGTCTCAAC	ACCCCAGGTA	TCACCACTGT	TATCGAGCCA	ATCATGACTC	400
G	L	N	T	P	G
L	N	T	P	G	I
GTGACCACAC	TGAAAAGATG	CTCAAGGTT	TTGGTGCTAA	CCITACCGTT	450
D	H	T	E	K	M
H	T	E	K	M	L
GAGACTGATG	CTGACGGTGT	CGGTACCATC	CGTCTTGAAG	GTCGTGGTAA	500
E	T	D	A	D	G
T	D	A	D	G	V
GCTCACCGGT	CAAGTGATTG	ATGTTCCAGG	TGATCCATCC	TCTACTGCTT	550
L	T	G	Q	V	I
T	G	Q	V	I	D
TCCCATTGGT	TGCTGCCTTG	CTTGGTCCAG	GTTCCGACGT	CACCATCCIT	600
P	L	V	A	A	L
L	V	A	A	L	L
AACGTTTGTA	TGAACCCAAC	CCGTACTGGT	CTCATCTTGA	CTCTGCAGTG	650
N	V	L	M	N	P
V	L	M	N	P	T

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Fig.10(3/4).

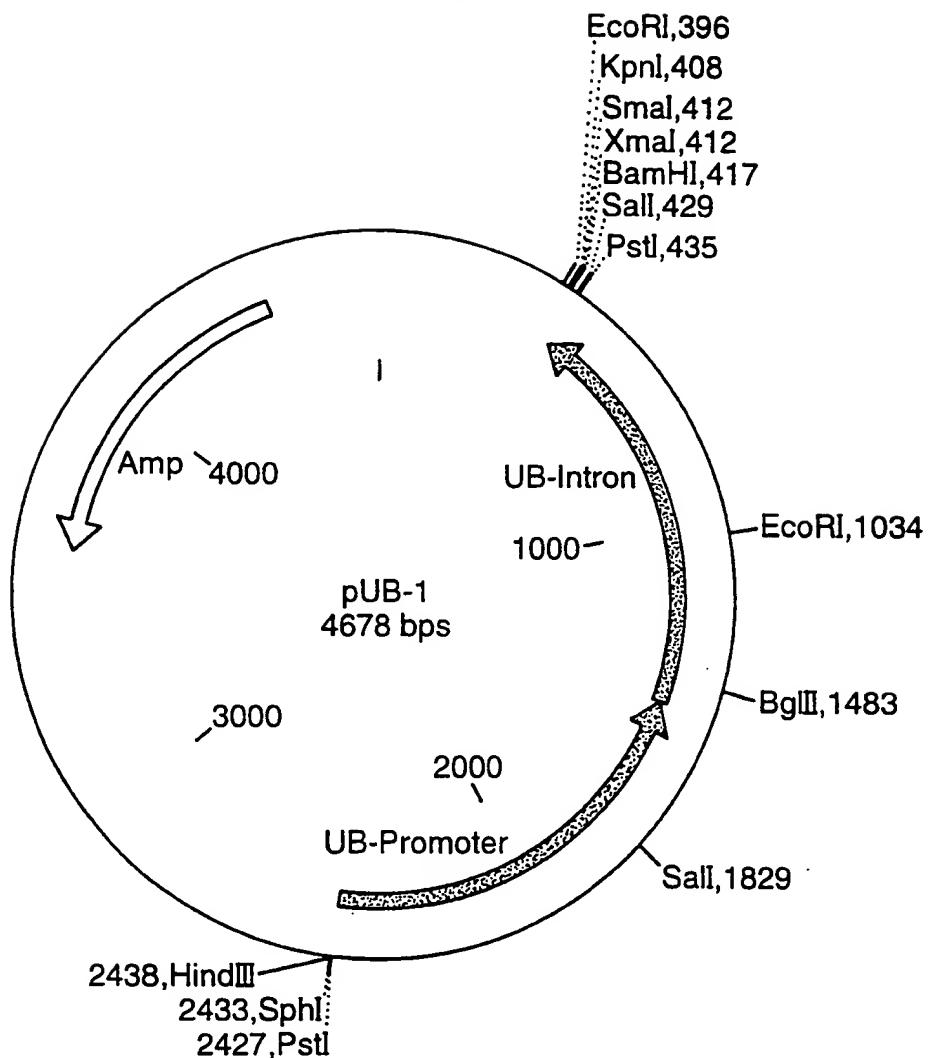
10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	660
TTGTGAATTTC					
C	E	F			

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## Fig.10(4/4).

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
AAGCTCTG	AGGAAATGGG	TGCCGACATC	GAAGTGTCA	ACCCACGTCT	50
K L L Q	E M G	A D I	E V I N	P R L	
TGCTGGTGG	GAAGACGTGG	CTGACTTGCG	TGTTCTGTTCT	TCTACTTTGA	100
A G G	E D V A	D L R	V R S	S T L K	
AGGGTGTAC	TGTTCCAGAA	GACCGTGCTC	CTTCTATGAT	CGACGAGTAT	150
G V T	V P E	D R A P	S M I	D E Y	
CCAATTCTCG	CTGTTGCAGC	TGCATTGCT	GAAGGTGCTA	CCGTTATGAA	200
P I L A	V A A	A F A	E G A T	V M N	
CGGTTGGAA	GAACCTCGTG	TTAAGGAAAG	CGACCGTCTT	TCTGCTGTGG	250
G L E	E L R V	K E S	D R L	S A V A	
CAAACGGTCT	CAAGCTAAC	GGTGTGATT	GCGATGAAGG	TGAGACTTCT	300
N G L	K L N	G V D C	D E G	E T S	
CTCGTCGTGC	GTGGTCGTCC	TGACGGTAAG	GGTCTCGGT	ACGCTTCTGG	350
L V V R	G R P	D G K	G L G N	A S G	
AGCAGCTGTC	GCTACCCACC	TCGATCACCG	TATCGCTATG	AGCTTCCCTCG	400
A A V	A T H L	D H R	I A M	S F L V	
TTATGGGTCT	CGTTCTGAA	AACCCTGTTA	CTGTTGATGA	TGCTACTATG	450
M G L	V S E	N P V T	V D D	A T M	
ATCGCTACTA	GCTTCCCAGA	GTTCATGGAT	TTGATGGCTG	GTCTTGGAGC	500
I A T S	F P E	F M D	L M A G	L G A	
TAAGATCGAA	CTCTCCGACA	CTAAGGCTGC	TTGATGAGCT	CGAATT	547
K I E	L S D T	K A A	.. A	R I	

Fig.11.



Ubiquitin promoter fragment PCR'd from maize.  
 2 Kb. fragment cloned into pUC19.  
 Junctions have been sequenced to confirm that it  
 is the Ubiquitin promoter.

Fig.12.

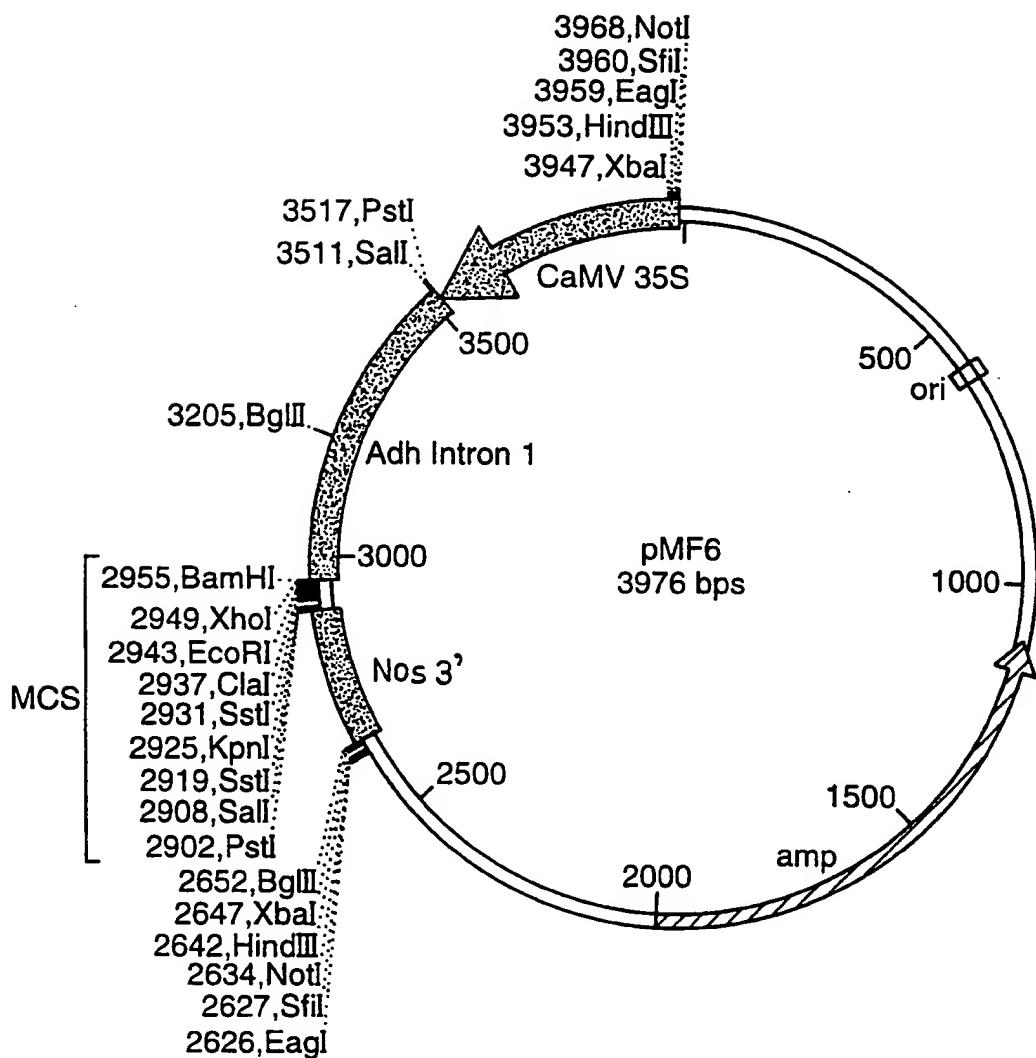


Fig.13.

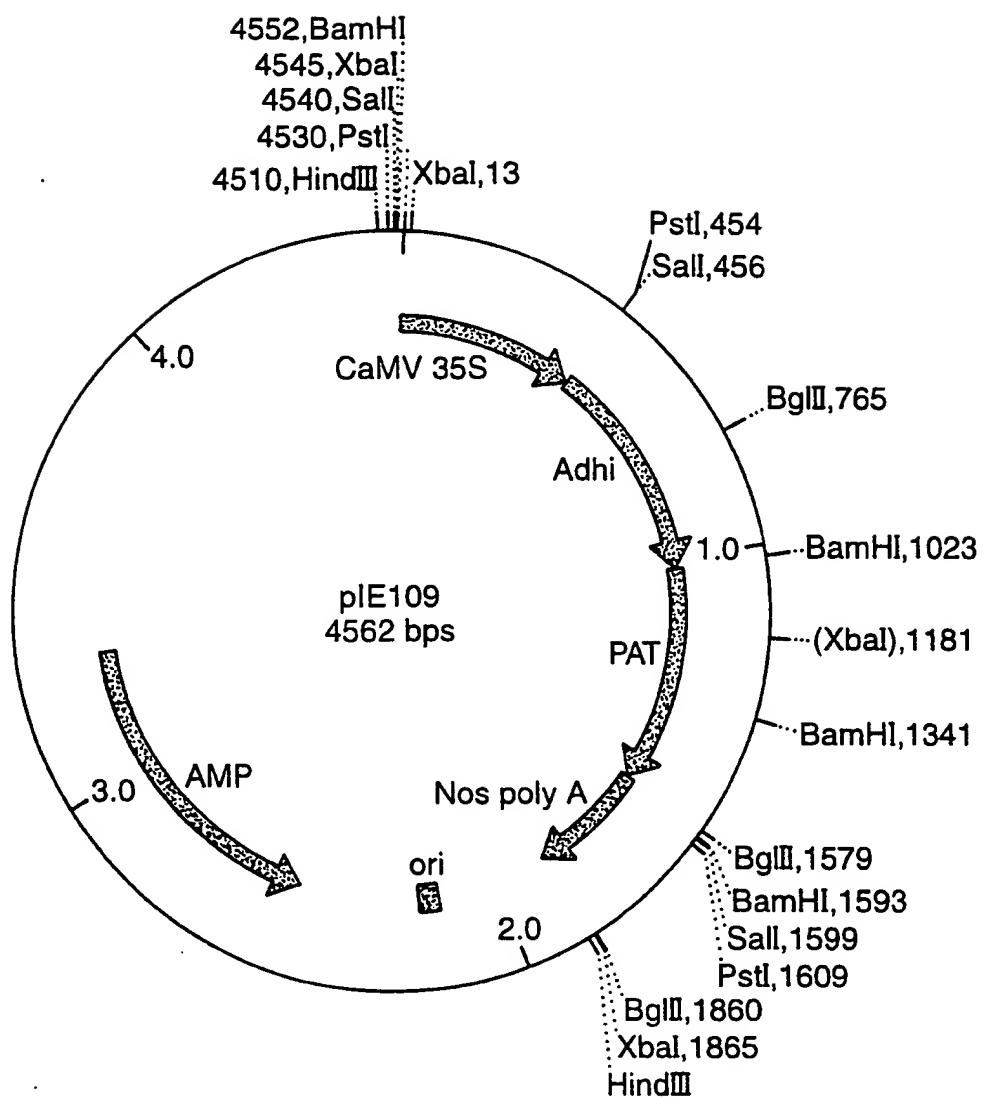


Fig. 14.

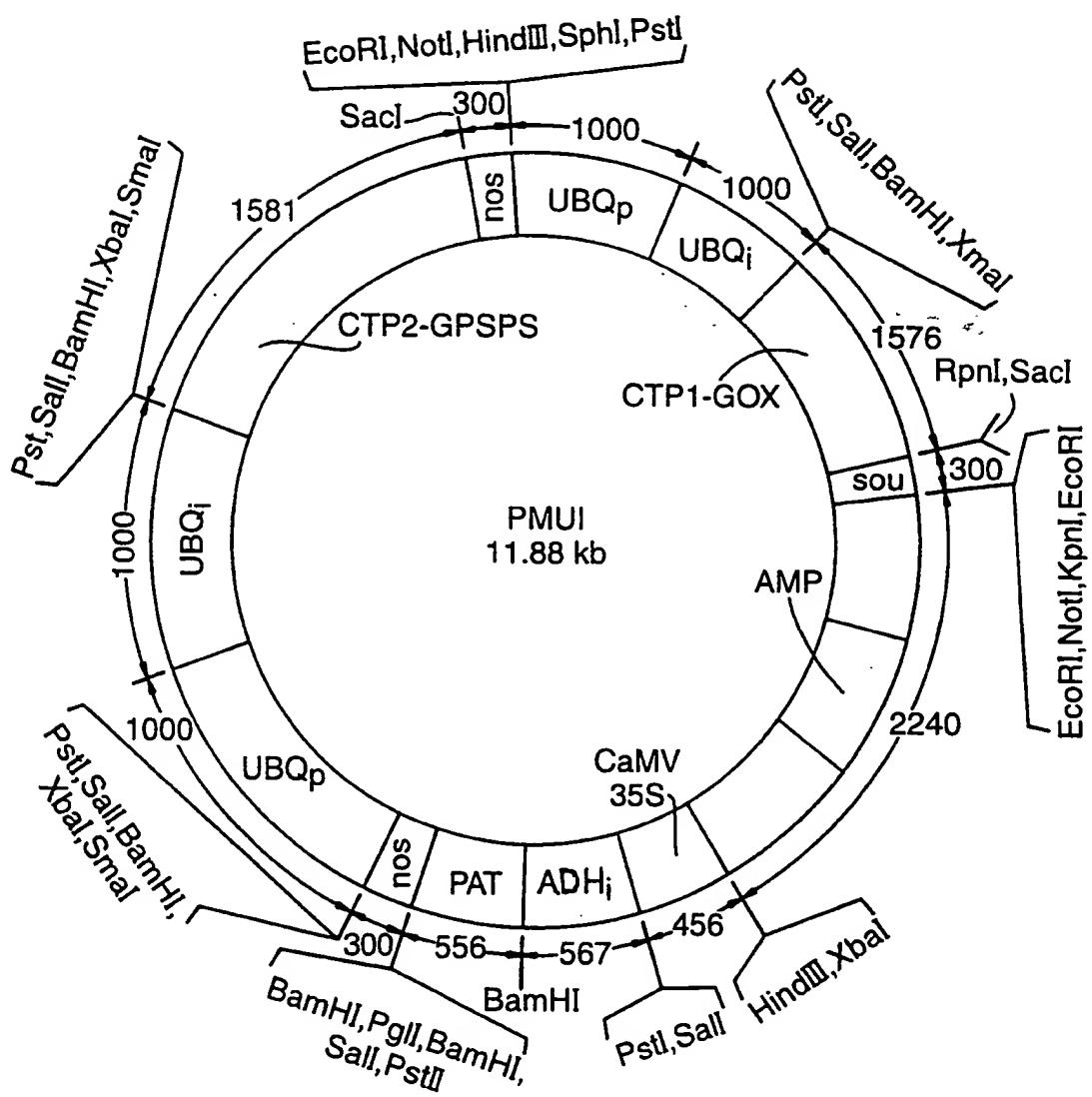


Fig.15.

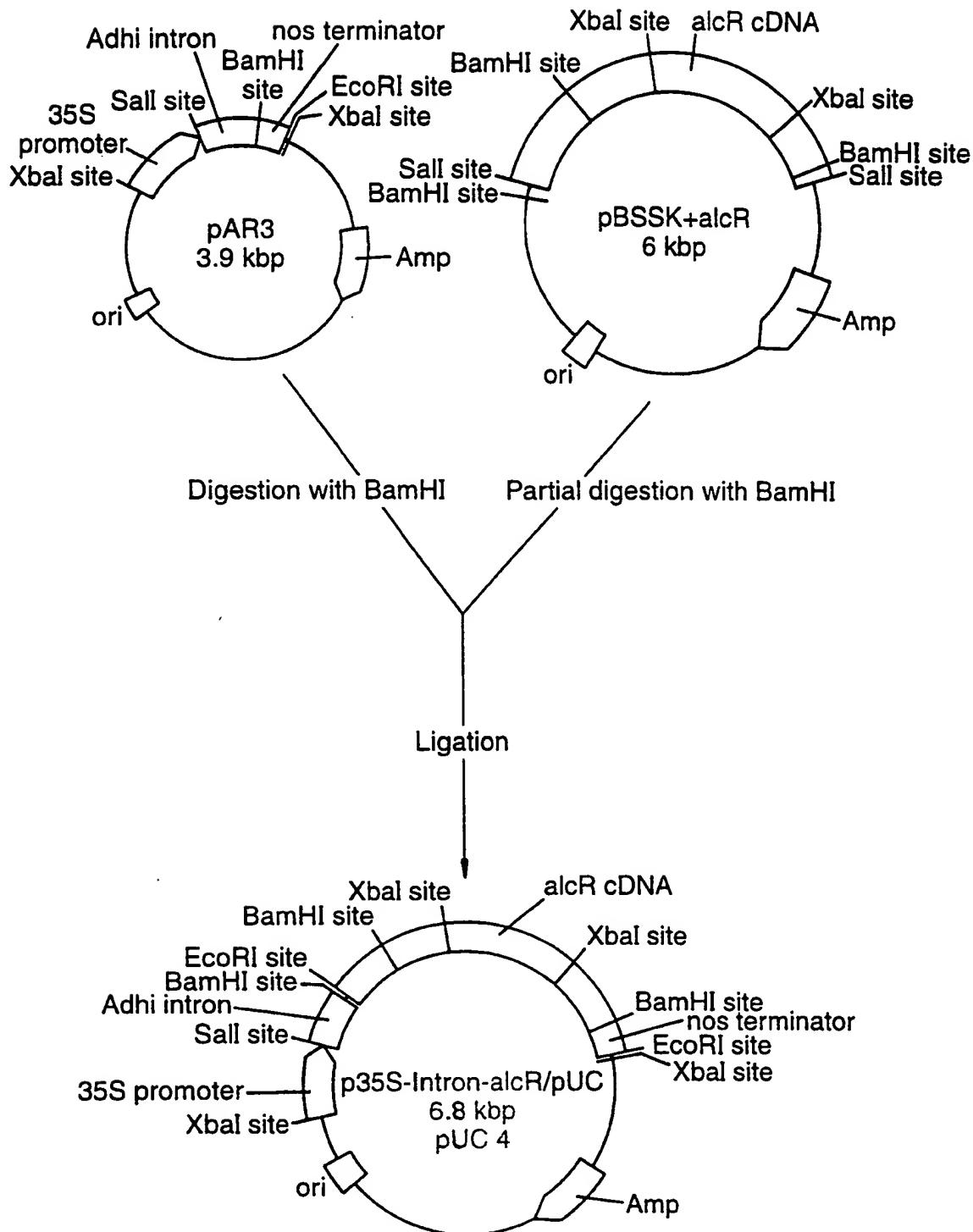
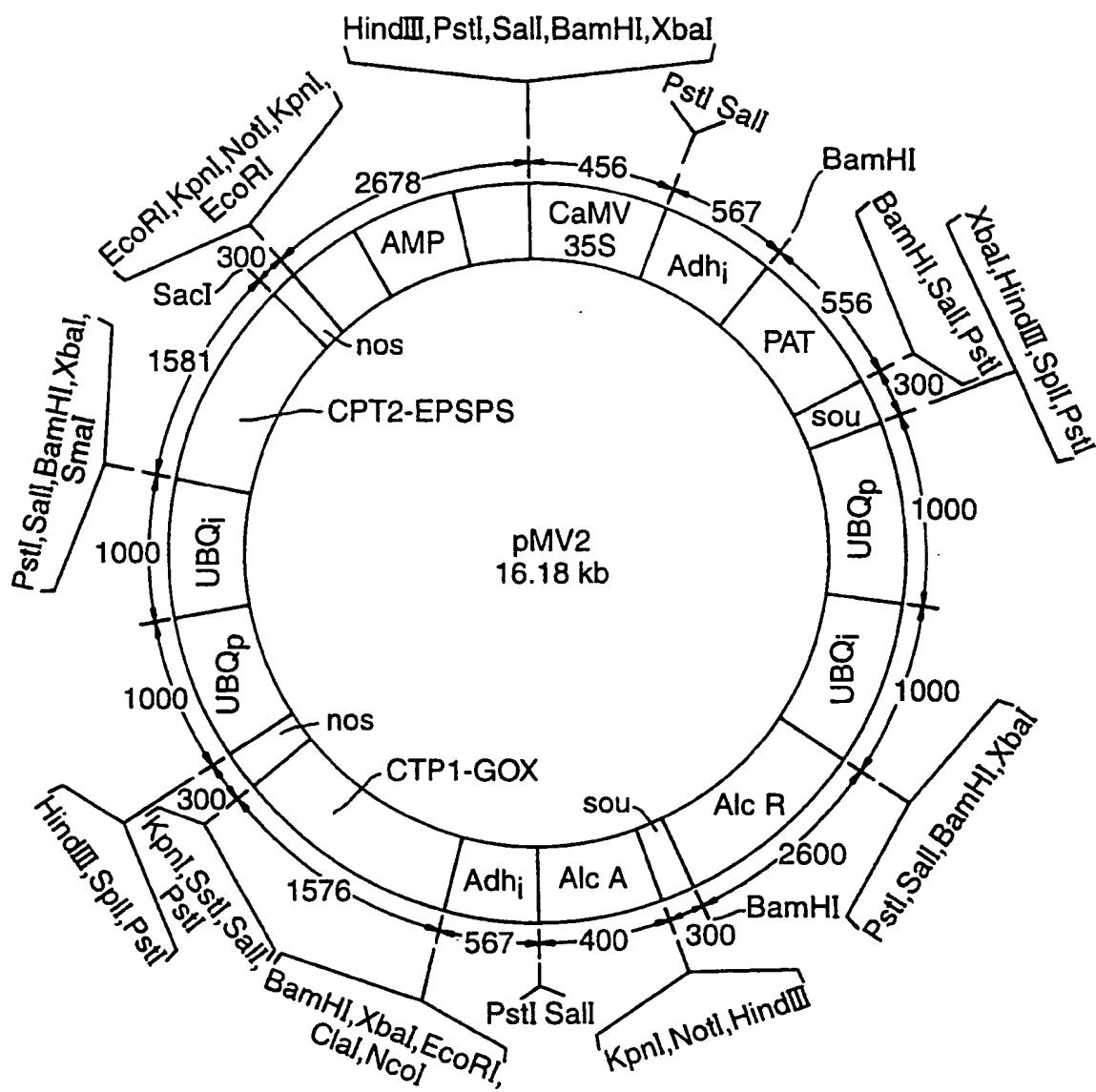


Fig.16.



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Fig.17.

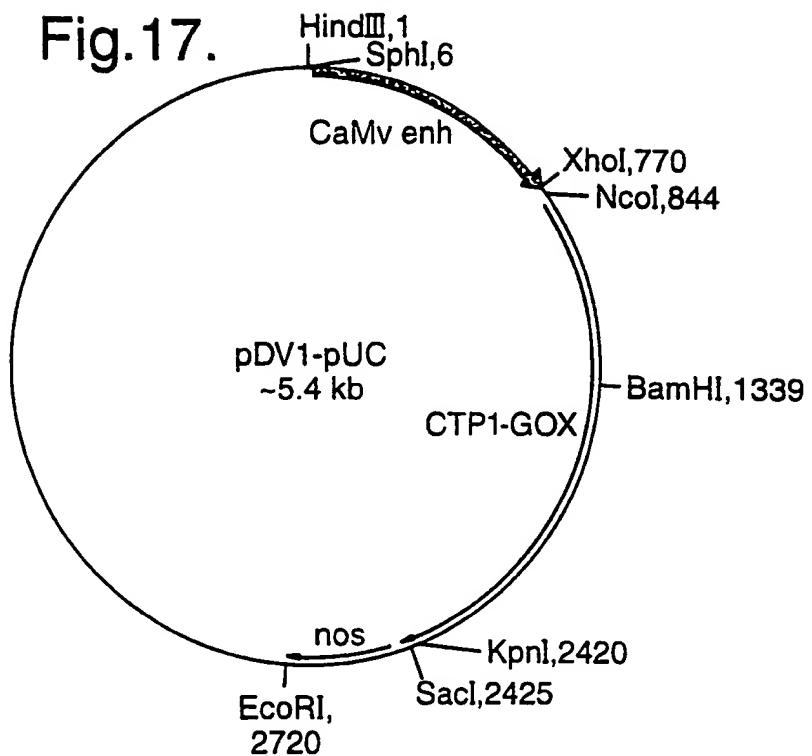


Fig.18.

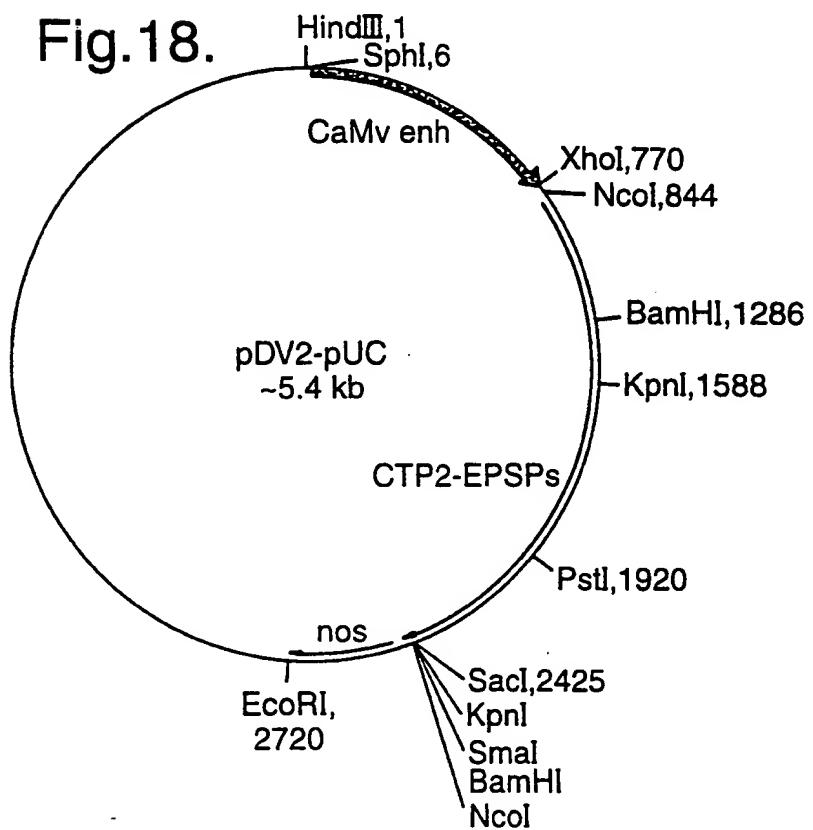
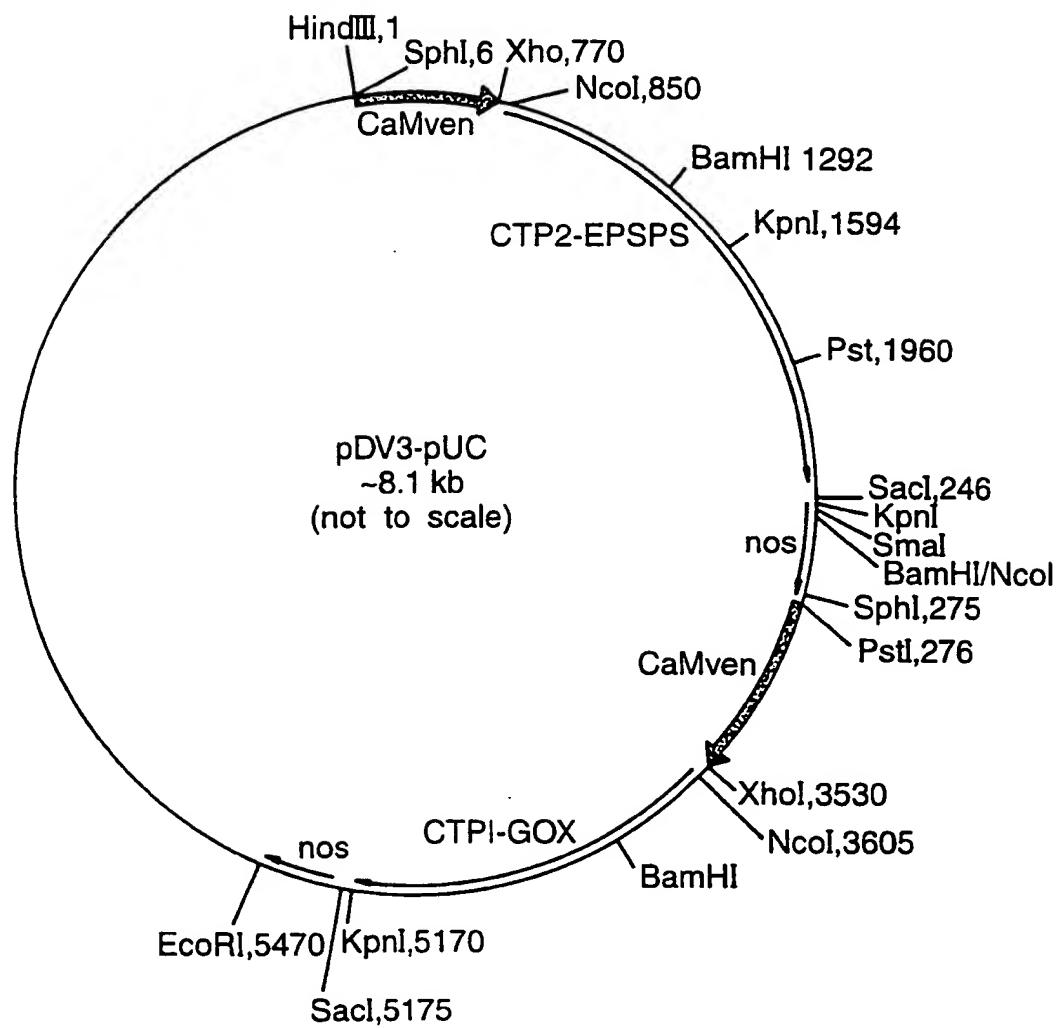


Fig.19.



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Fig.20.

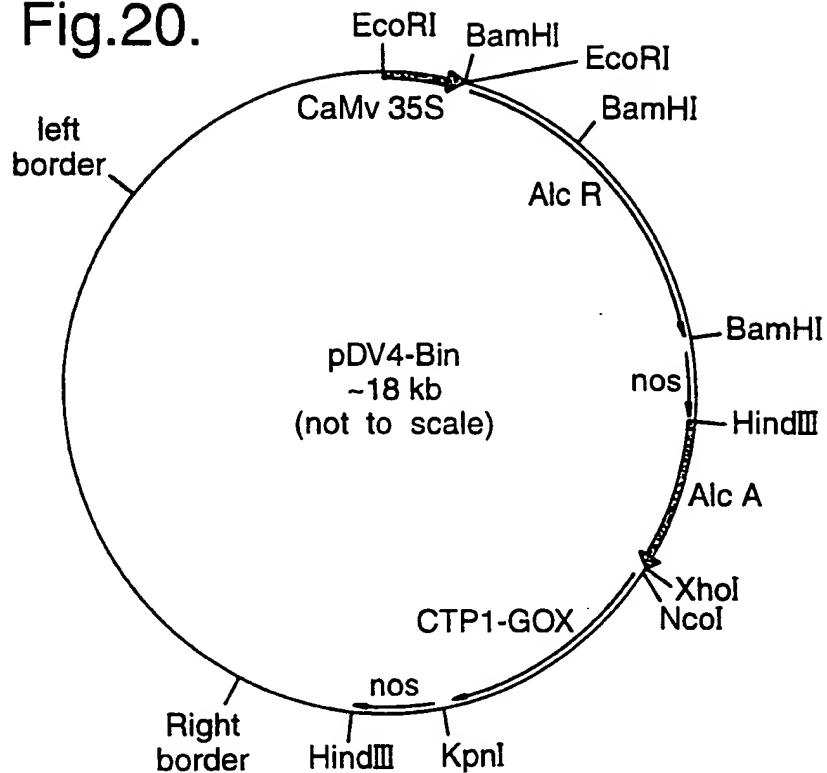
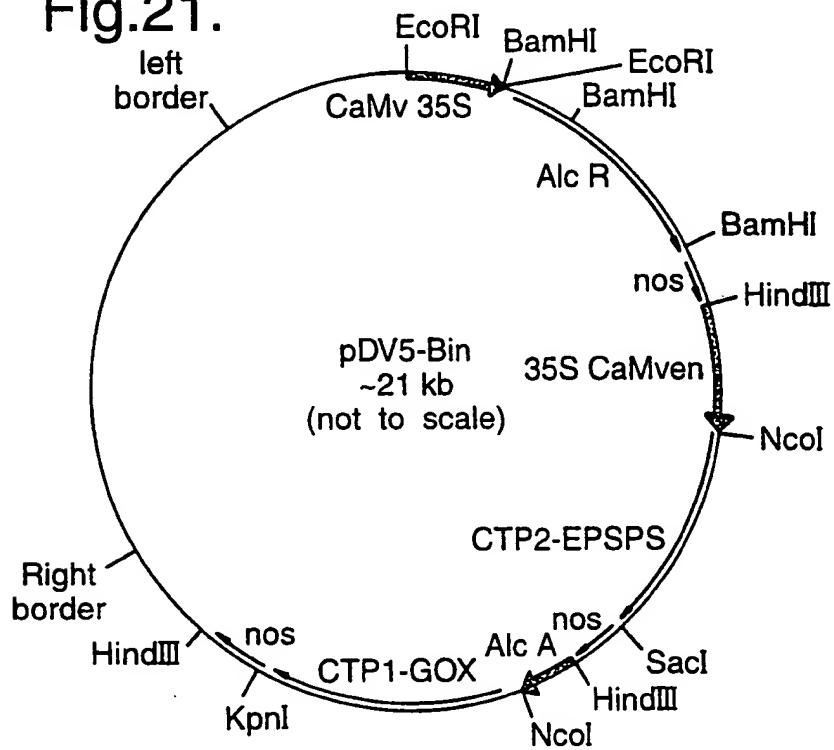
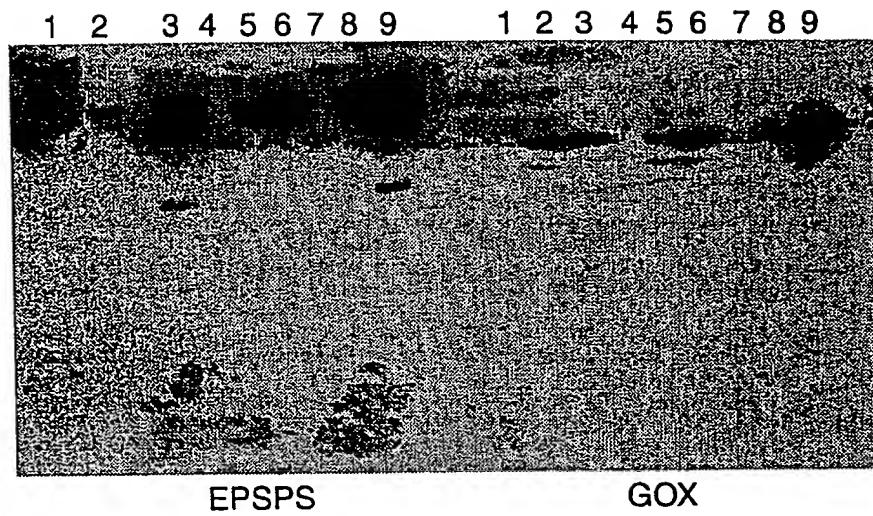


Fig.21.



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Fig.22.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/96/01883

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 388 186 (DU PONT) 19 September 1990	1,3, 7-12,14, 15
Y	see page 10, line 35 - line 41 see page 78, line 1 - page 82, line 40 ---	3
Y	WO,A,93 01294 (ICI PLC) 21 January 1993 see page 11, line 16 - page 12, line 2 ---	3
X	WO,A,93 05164 (UNIV LEICESTER) 18 March 1993 see page 54, line 10 - line 18 see page 48, line 1 - line 19 ---	1,3,8-12
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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\*&\* document member of the same patent family

1 Date of the actual completion of the international search  15 January 1997	Date of mailing of the international search report  29.01.97
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Fax: (+ 31-70) 340-3016

Authorized officer

Maddox, A

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/01883

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 332 104 (CIBA-GEIGY) 13 September 1989 see page 17, line 25 - line 29 see page 22, line 41 - line 44 see page 36, line 55 - page 38, line 27 see page 58, line 30 - page 59, line 16 see page 40, line 50 - page 41, line 40 ---	1,3, 8-12,14, 15
X	BIOTECHNOLOGY, vol. 5, no. 7, July 1987, pages 726-730, XP002022728 FILLATI, J.J., ET AL.: "Efficient transfer of a glyphosate tolerance gene into tomato using a binary agrobacterium tumefaciens vector" see page 729, right-hand column ---	1,2,7-14
A	WO,A,90 13658 (UNIV ALBERTA ;SZALAY ALADAR A (CA); LANGRIDGE WILLIAM A R (CA)) 15 November 1990 see the whole document ---	7
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E	WO,A,96 25505 (PIONEER HI BRED INT) 22 August 1996 see examples 4,5 -----	1,3,8-13

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/GB 96/01883

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